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THE IMMUNOGLOBULINS OF MICE: ETHANOL FRACTIONATION AND DETECTION OF HOMOREACTANT

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THE IMMUNOGLOBULINS OF MICE: ETHANOL FRACTIONATION AND DETECTION OF HOMOREACTANT

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CHAPTER I

INTRODUCTION

Antibodies belong to a heterogenous, but structurally related, group of proteins called immunoglobulins. The functional role of antibodies is markedly influenced by molecular characteristics which are dependent of antigen-binding activity. Differences in skin fixation, rate of catabolism, complement-binding activity, placental transfer and genetically determined antigens reflect specific characteristics of different classes of immunoglobulins (8).

New classes of immunoglobulins have recently been characterized in the mouse, and major classes divided into subclasses. The five immunoglobulin classes of mice have been defined as 7SY1, 7SY2a, 7SY2b, YA and YM globulins (10, 11). To avoid confusion, they have recently been named YF, YG, YH, YA, and YM, respectively (36). Prior to a complete study of the biological functions of the individual

classes of mouse immunoglobulins, techniques must be available which will consistently yield relatively large amounts of pure product.

Several methods for purifying immunoglobulins have been developed (1, 13, 23, 32, 33), but many of the procedures have limitations that make them unsatisfactory for general use (9, 40). The majority of the methods use DEAE-cellulose as a major step in the procedure (32); however, mouse serum is not fully separated due to its adherence to the adsorbent and its subsequent elution with transferin (39). To accomplish fractionation, Fahey et al. (10) have used zone electrophoresis in conjunction with gel chromatography and ion-exchange chromatography in a three step procedure. Although results were excellent, the procedure was complicated and yields of immunoglobulin were low. Precipitation with neutral salts (12) have also been studied. The results were variable and the products were contaminated with other components of the serum. Recently, a two step procedure involving zone electrophoresis and isoelectric focusing has shown promise, but only YH immunoglobulin has been purified (27).

The separation of the components of biological tissues and fluids by ethanol fractionation was carried out by Cohn et al. (2). This method was directed to the isolation and recovery of all recognizable components of the system in question. There are advantages to alcohol

precipitation methods from a theoretical and practical viewpoint. From a practical viewpoint, a protein separated from a concentrated salt solution must be freed of salts precipitated with it. This is necessary since the substances used in the salting-out are usually not desirable when the protein preparation is to be used chemically. From a theoretical view, the highly specific forces that determine the interactions between proteins and electrolytes at low ionic strength are masked in concentrated salt solutions.

Nichol and Deutsch (28) developed an alcohol precipitation method to purify globulins from the animal serum. They were able to purify $\gamma 2$-immunoglobulins from the serum of the goat, dog, rabbit, rat, chicken, and guinea pig. It was found in the individual species that the chemical treatment varied, but in all cases there was an initial and important step in which the antibody-rich γ-globulins were precipitated from a diluted serum. The previous treatment was followed by a purification treatment(s) to remove certain small amounts of contaminating beta globulins. In this way, the γ-globulins were obtained in a relatively pure form. The methods involved variation in ethanol, salt concentration and pH, as used in studies on human γ-globulins (3, 4).

The ethanol fractionation of mouse serum has not been reported. A portion of this study was designed to develop ethanol fractionation techniques in which mouse

serum could be rapidly purified into its Y-globulin components.

In 1965, Mandy et al. (18) described a new serum factor in normal rabbits which was termed homoreactant (HR). The presence of this factor in rabbits and humans has been well established (16, 19, 20, 21, 22). HR was described as a heat stable, mercaptoethanol resistant and nondializable serum factor in normal rabbits which was capable of causing agglutination of human red cells sensitized with the 3.5S (Fab) univalent fragments of rabbit anti-human erythrocyte antibody (18). Subsequent studies have shown that HR is a YG-globulin as determined by DEAE-cellulose chromatography and zone electrophoresis (19).

HR differs from anti-antibody, rheumatoid factor and anti-allotype antibody. Anti-antibody is a factor in certain human sera that agglutinates O Rh(+) cells "sensitized" with incomplete anti-Rh antibodies but is not inhibited by normal or immune human serum (24). Anti-antibody differs from HR since it can be absorbed by "sensitized" erythrocytes (18). On the other hand, rheumatoid factor differs from HR since rheumatoid factor can be inhibited by normal human serum (25). Isoantibodies which are directed against Y-globulins of genetic types (allotypes) other than that of the recipient have been produced in the rabbit (3) and in mice (4). HR differs from anti-allotypic

antibody since it has been found in all normal rabbits tested (18).

Subsequent investigations have shown that HR was specifically directed to sites on the Fab fragment of the γ-globulin which were ordinarily buried on the intact γG-globulin molecule and that HR was a 7S naturally occurring antibody (20). Studies with the $(Fab')_2$ fragments (pepsin treated HR) demonstrated that they were good inhibitors of native HR activity (20). Furthermore, pepsin digestion of γG-globulin containing HR exposed sites which were recognized by native HR, resulted in immediate neutralization of Fab' or $(Fab')_2$ specificity. This implies that HR may possess autospecificity (20, 21).

The biological significance of HR is of obvious interest to investigators of autoimmune reactions since HR reacts with autologous as well as homologous fragments (22). This also appears to be true for the human counterpart of HR (16). It has been suggested that the natural antibodies to hidden determinants serve the animal in some capacity, such as clearance of catabolic products of γG-globulins (42).

The presence of HR in mice has not been reported. A portion of this study was designed to demonstrate the presence of HR in mice and to investigate the possibilities of strain differences in HR titer.

Some of the points which were considered in this study are given below: 1. Could mouse YG and YH globulins be purified by ethanol fractionation techniques? Could YF, YA, and YM mouse globulins be purified in simple steps using by-products of ethanol fractionation? 3. Were these procedures significantly easier to perform than previous methods and could pure preparations be obtained using small amounts of material? 4. Was HR present in mice? How did HR titer vary with mouse strain?

(In this study, the terminology of Potter and Lieberman (36) will be used when ever possible. In cases where YH and YG are combined, the term 7SY2 will be employed.)

CHAPTER II

MATERIALS AND METHODS

Animals Used. The mice used were Swiss hybrids, C57BL/6J and CBA/J which are maintained in our laboratory. Twelve week old mice of both sexes were used.

New Zealand white rabbits weighing 2-3 kg were used for the production of all antisera used in this study.

Normal Mouse Serum. Mice were bled by exsanguination and sera from 10 mice were pooled for testing. The sera samples were maintained at -20 C until used. All sera used in HR studies were heated at 56 C for 20 min and absorbed with O Rh(+) erythrocytes to remove heteroagglutinins.

Ethanol Fractionation. Ethanol fractionation was followed using the general scheme of Nichol and Deutsch (28). Ninety-five per cent ethanol was diluted to 50% in all cases before addition to protein solutions. In all steps, an International PR-2 centrifuge (International Centrifuge Co.) was used for the separation of the fractions.

1. Primary fractionation. The first fraction was obtained by diluting 1 volume of mouse serum with 3 volumes of distilled water. The pH was adjusted to 7.7 with 0.025 M disodium phosphate. The misture was cooled to 0 C and brought to a final ethanol concentration of 20%. The mixture was stirred for 12 hr and centrifuged at 1500 x g for 30 min. Samples were taken at various time intervals during the stirring procedures to determine the degree of fractionation.

2. Secondary fractionation. The primary precipitate (ppt A) was suspended in 0.01 M sodium chloride to give a 0.75% protein suspension (w/v). Alliquots were adjusted to pH 4.9-5.2 with 0.05 acetic acid. The temperature was maintained at -2 C and the alliquots were adjusted to 0-15% alcohol concentration. The ionic strength was adjusted from 0.005-0.010. The mixture was stirred for 6 hr and centrifuged at 1800 x g for 1 hr.

3. Subfractionation of precipitate B. The secondary precipitate (ppt B) was resuspended in distilled water at 0 C and the pH adjusted to 5.1. The solution was filtered in the cold and the filtrate adjusted to pH 5.1 with 0.05 M disodium phosphate. The mixture was brought to a 20% ethanol concentration and stirred at -2 C for 2 hr. The precipitate was removed by centrifugation at 1800 x g for 30 min and labelled precipitate BB (ppt BB).

4. Subfractionation of supernant B. The supernatant to precipitate B was adjusted to pH 5.6-6.0 with 0.025 M disodium phosphate and the alcohol concentration brought to 12%. The temperature was maintained at -3.5 C and the ionic strength at 0.005-0.010. The precipitate (ppt C-1) was removed by centrifugation at 2400 x g after 12 hr of stirring.

5. Subfractionation of supernatant C-1. The supernatant to ppt C-1 was adjusted to pH 7.2-7.4 with 0.025 M disodium phosphate and the ethanol concentration was brought to 25% at -10 C. The ionic strength was adjusted from 0.01 to 0.015. The mixture was shaken at -10 C for 10 hr and the final precipitate (ppt C-2) was collected by centrifugation at 2400 x g for 2 hr.

A complete explanation of the method is shown in Figure 1.

Electrophoresis.

1. Cellulose acetate membrane (CAM) electrophoresis. Normal mouse serum, ethanol fractions of mouse serum or ammonium sulfate fractions of mouse serum were separated by CAM electrophoresis on a Microzone Electrophoresis Chamber (Beckman Instruments). CAM strips were soaked in pH 8.6 barbital buffer (u=0.075) for 10 min. The strips were blotted and immediately fixed in the cell. Duplicate samples of 25 ul were placed midway from the ends of the cell strip. The electrophoresis was performed

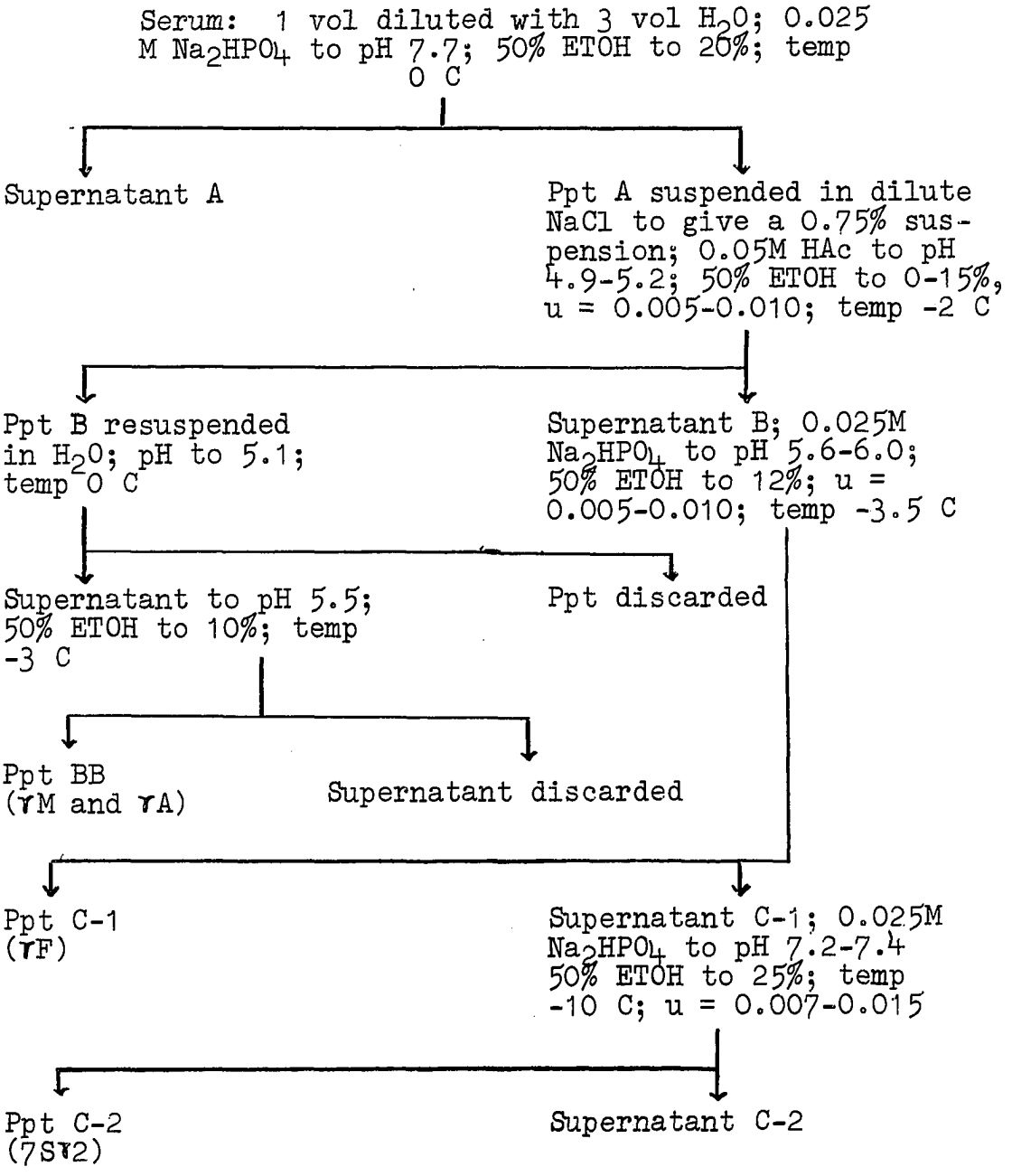

Figure 1. General scheme for the fractionation of mouse serum.

using 250 V for 30 min. The strips were fixed in 20% sulfosalicylic acid for 10 min and stained in 2% Coomassie Brillant Blue (Fischer Scientific) for 30 min. The strips were washed in distilled water for 1 hr and air dryed between 2 sheets of filter paper. The strips were scanned on a Densicord Electrophoresis Scanner (W. H. Curtain) using an expansion drive of 4:1 and an absorption filter of 610 mu.

2. Zone electrophoresis. Normal mouse serum or fractions of mouse serum were separated in potato starch with modifications of the method of Osterland (29). The protein was dialized against pH 8.6 barbital buffer (μ= 0.05) and then applied to a 3 x 1 cm trough in a bed of potato starch (6 x 48 x 1 or 3 x 30 x 0.5) soaked in the buffer. The electrophoresis was carried out in the cold for 23 hr using a potential gradient of 3.5 V/cm. After electrophoresis, the block was removed and 1 cm sections were cut. Elution of the protein from the segments was performed by addition of 10 ml of the saline to each block with subsequent centrifugation at 500 x g. The amount of protein in each segment was determined by the method of Lowry (17) as described.

Gel choromatography. Normal mouse serum of fractions of mouse serum were dialized against 0.1 M Tris-buffer containing 0.2 M sodium chloride, pH 8.0 (14). The protein was applied to a 2.5 x 50 cm column containing 10.5 g of

Sephadex G200 (Pharmacia Fine Chemicals) in the buffer. Buffer was applied to obtain a flow rate of 12 ml/hr. Effluent fractions of 5.0 ml were collected and the protein distribution determined by measurement at 280 mu on a DB-G Spectrophotometer (Beckman Instruments).

Ion-exchange chromatography. Ethanol fractions of mouse serum were separated by chromatography on diethylaminoethyl-Sephadex A50(DEAE-Sephadex) with modifications of the method of Fahey et al. (9). A linear gradient was used from 0.01 M to 0.30 M phosphate pH 8.0 buffers to elute the proteins from a 2.5 x 25 cm column. The distribution of the protein was determined by measurements at 280 mu as described.

Protein determination.

1. Ultraviolet (UV) determination. Protein concentrations were estimated from the optical density (OD) of the solution in saline at 280 mu using an extinction coefficient of 15 for a 1% solution (38).

2. Lowry determinations. Protein concentrations were determined by modifications of the method of Lowry et al. (17) when protein absorption at 280 mu was impractical. To 0.3 ml of sample, 2.5 ml of alkaline copper solution was added. The alkaline copper solution consisted of 50 parts of 2% sodium carbonate in 0.1 N sodium hydroxide to 1 part of 0.5% copper sulfate in 1% sodium tartrate. After standing for 15 min at room temperature,

0.25 ml of phenol reagent, diluted 1:2, was added. The tubes were heated at 56 C for 15 min and cooled until read at 660 mu.

Erythrocytes. Whole blood was obtained from O Rh(+) donors, mixed with an equal volume of Alsever's solution and stored at 4 C. Cells were discarded after 14 days.

Production of anti-human erythrocyte antisera (anti-HEA). Antisera to human erythrocytes was prepared by injecting three strains of mice intravenously (iv) or intraperitoneally (ip) with 0.1 ml (1.8×10^8 cells) of a saline suspension of washed human O Rh(+) erythrocytes. Booster injections of 1.8×10^8 cells were given at weekly intervals to selected groups. The mice were bled by decapitation at various intervals. The sera of each group of 10 mice were pooled and heated at 56 C for 20 min. The γG-globulin fractions of the anti-HEA antisera were obtained by ammonium sulfate fractionation at 40% saturation (23). Each γG-preparation was tested for homogencity by CAM electrophoresis as previously described. A complete schedule of injections is given in Table 1.

Rabbit anti-mouse globulin(anti-M G). Antiserum to mouse γG-globulins was prepared by injecting rabbits ip with an alum precipitate containing 30 mg of mouse γG-globulin once weekly for 4 weeks. The rabbits were then bled weekly by cardiac puncture and the titer boostered by

Table 1. Schedule for injection of mice with 1.8×10^8 human O Rh(+) cells.

Group No.*	Strain	Day injected	Route injection	Day bled
1	Swiss	1	iv	1
2	Swiss	1	iv	5
3	Swiss	1	iv	9
4	Swiss	1	iv	13
5	Swiss	1	iv	17
6	Swiss	1	iv	25
7	Swiss	1	ip	1
8	Swiss	1	ip	5
9	Swiss	1	ip	9
10	Swiss	1	ip	13
11	Swiss	1	ip	17
12	Swiss	1	ip	25
13	Swiss	1,7,14	iv	21
14	Swiss	1,7,14	ip	21
15	Swiss	1,7,14	iv,iv,ip	25
16	C57 BL/6J	1,7,14,21	ip	21
17	C57 BL/6J	1,7,14	iv,iv,ip	21
18	CBA/J	1,7,14,21	ip	25
19	CBA/J	1,7,14	iv,iv,ip	21

*10 mice per group

subcutaneous (sc) injections of 10 mg of the antigen in saline. The antiserum was heated at 56 C for 20 min and absorbed with O Rh(+) erythrocytes.

Preparation of 3.5S univalent(Fab) anti-HEA. The Fab of mouse 7S anti-Hea was prepared and purified according to the procedure of Porter (34) and Petermann (31). The 7S γG-globulin preparation in 0.1 M phosphate buffer, pH 7.5, and 0.002 ethylenediaminotetracetic acid (EDTA) was treated with 0.03 M cycteine for 2 hr at 37 C. Mercuripapain produced by the method of Kimmel and Smith (15) in an amount of 1% by weight of protein was added to groups 13-19 and the incubation was allowed to continue at 37 C for 16 hr. Groups 1-12 were treated in a similar manner except soluble papain was used instead of mercuipapain. In both cases, the papain was inactivated by dialysis against distilled water for 2 days at 4 C.

The fractionated globulin was purified by passing the material through a column (1 x 18 cm) packed with Sephadex G100 and eluted with Tris-buffer, pH 8.0, containing 0.2 M sodium chloride. The protein obtained in the void volume was considered to be undigested protein. The fractions containing protein obtained after the void volume were pooled and concentrated by pervaporation.

The fractions were dialyzed against barbital buffer, pH 8.6(u = 0.05) and separated by starch block electrophoresis as previously described. One cm sections of starch

were eluted with 5 ml of saline and the protein determined by the method of Lowry (17) as described above. The most cathodal of the protein containing segments were taken to contain purified Fab fragments (29). The anodal protein segments were crystallized for Fc fragments by dialysis against 0.1 M sodium acetate (35). The segments containing Fab fragments were pooled and pervaporated to the original volume. The Fab fragments used in inhibition studies were prepared from normal mouse γG-globulin by the same procedure.

Serological methods.

1. Sensitization. Two per cent suspensions of human O Rh(+) erythrocytes were sensitized by incubation with equal volumes of two-fold dilutions of the Fab anti-HEA at 37 C for 1 hr in lucite V-shaped bottom Microtiter plates (Cooke Engineering Co.). Phosphate buffered saline (PBS), pH 7.2, was used as the diluent. The cells were resuspended after sensitization by shaking on a Yankee Rotary Shaker (Clay-Adams) for 10 min.

2. Agglutination. Agglutination was performed by adding 0.025 ml of serial twofold dilutions of normal or ethanol fractionated mouse sera to 0.050 ml alliquots of the sensitized or unsensitized cells in the plates. The cells were shaken for 10 min and allowed to stand at room temperature for 2 1/2 hr. The results were scored from 0 to 4+. Appropriate controls were included in each experiment.

3. Tests for mercaptoethanol (ME) sensitivity. Equal volumes (0.025 ml) of serial twofold dilutions of pooled normal mouse serum or mouse immunoglobulin fractions are varying concentrations of ME (Matheson, Coleman, and Bell) were incubated at room temperature for 1 hr. Sensitized cells were added and agglutination test performed as described.

4. Inhibition of agglutination. Varying amounts of Fab fragments of normal mouse ϒG-globulin were added to dilutions of pooled normal mouse sera. Each mixture was incubated at 37 C for 30 min and then tested for HR activity by the addition of sensitized red cells.

CHAPTER III

RESULTS

Evaluation of ethanol fractionation of mouse serum.

1. Primary fractionation. It was found that the primary fractionation was successful in removing 97% of the gamma globulins from mouse serum with less than 2% contamination as analyzed by CAM electrophoresis (Figure 2), zone electrophoresis (Figure 3) and gel filtration (Figure 4). It was found that fractionation was time dependent with the successful degree of fractionation improving with time, up to 12 hr, as seen in Table 2.

2. Secondary fractionation. The secondary fractionation step was found to be the most critical step in the procedure, since there was separation of γM and γA from γF and 7Sγ2 globulins (28). Not only was this step dependent on pH, but also alcohol concentration and ionic strength. The best parameters, determined by CAM electrophoresis and per cent yield, was found to be pH 5.1, 5% alcohol and 0.010 ionic strength. A complete analysis is shown in Tables 3 and 4.

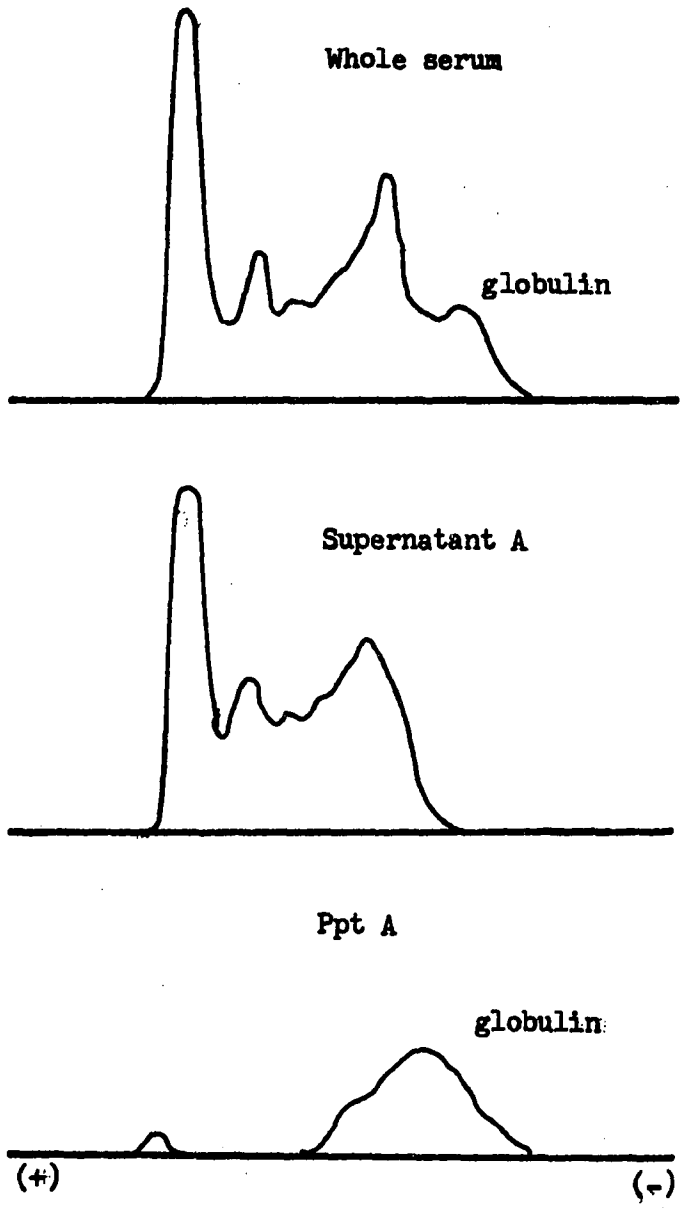

Figure 2. Primary fractionation of whole mouse serum as analyzed by CAM electrophoresis.

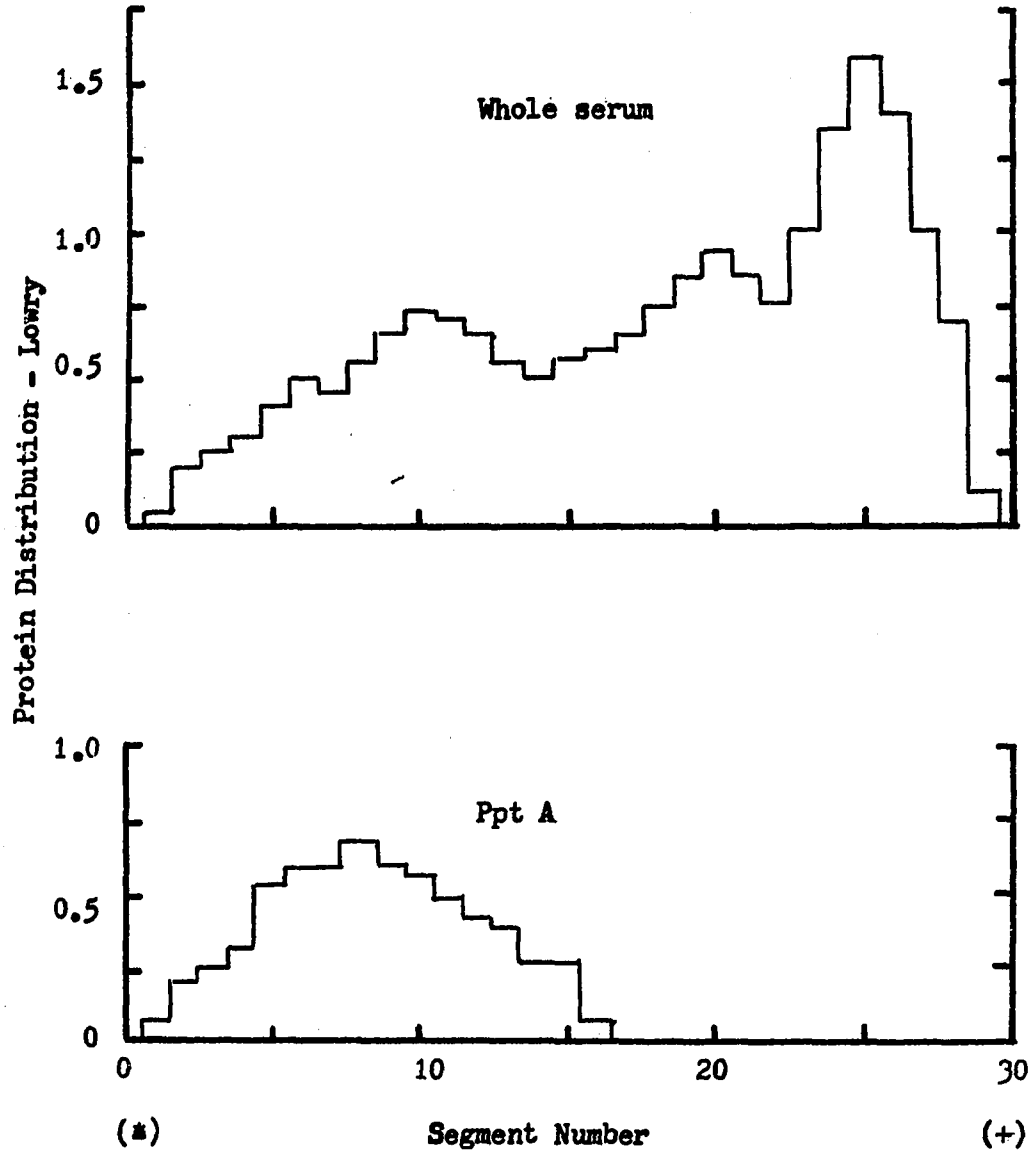

Figure 3. Zone electrophoretic distribution of mouse serum and ppt A of ethanol fractionation.

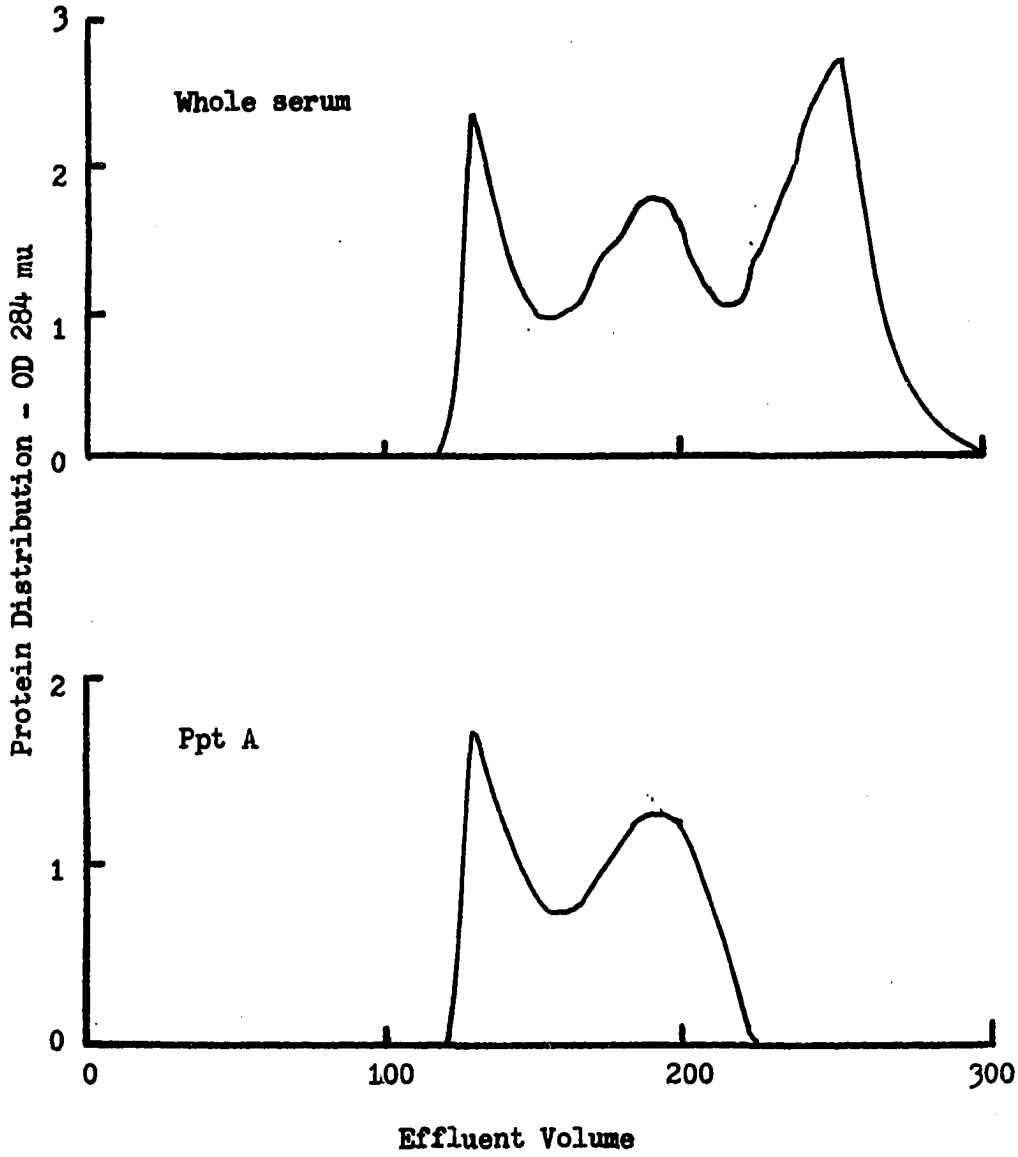

Figure 4. Gel filtration of whole serum and ppt A on Sephadex G200.

Table 2. Primary separation of mouse serum gamma globulins against time as assayed by CAM electrophoresis.

Sample time after ethanol addition (hrs)	%γ-globulins precipitated	% impurities*
1/2	50	17
1	62	13
2	69	11
4	86	9
8	94	5
12	97	2

*Albumin, alpha globulins, beta globulins

Table 3. Effect of pH and per cent alcohol on secondary fractionation.

Sample No.	pH	% alcohol	Yield final ppt*
1	4.9	15	0.21
2	4.9	10	0.39
3	4.9	5	0.47
4	4.9	0	0.50
5	5.0	15	0.54
6	5.0	10	0.69
7	5.0	5	0.71
8	5.0	0	0.77
9	5.1	15	0.82
10	5.1	10	0.96
11	5.1	5	1.00
12	5.1	0	0.67
13	5.2	15	0.53
14	5.2	10	0.48
15	5.2	5	0.28
16	5.2	0	0.16

*7S72 final ppt, defined in ratio of yield/ maximal yield.

Table 4. Effect of ionic strength on secondary fractionation.

Ionic strength	Yield final ppt*
0.0050	0.77
0.0750	0.83
0.0100	1.00
0.0125	0.93
0.0150	0.81

*7Sγ2 globulin as final ppt, defined in ratio of yield/maximal yield.

3. Subfractionation of precipitate B. The subfractionation step was found to be effective in purification of γM and γA from remaining contaminants. However, the yield of γM and γA in precipitate BB was smaller (53%) than would be expected based on the results of Fahey et al. (10). Separation of γM and γA were attempted by zone electrophoresis, gel chromatography, and ion-exchange chromotography. Zone electrophoresis gave one continual protein pattern (Figure 5). However, when the anodal and cathodal segments were subjected to gel filtration, purity was established (Figure 6). Likewise, gel filtration of precipitate BB was effective in separation of γA and γM (Figure 6). Ion-exchange chromatography was not effective in separation (Figure 7).

4. Subfractionation of supernatant B. This step was added to the procedure to remove the γF globulins from the 7Sγ2 globulins. Although the pH range (5.6-6.0) did not seem to be too important, the best yield of γF globulin was obtained at pH 5.8 (Table 5). Ionic strength was found to be most effective at 0.010 (Table 6). The purity of the fraction was established by CAM electrophoresis, zone electrophoresis (Figure 8) and gel chromatography (Figure 9).

5. Subfractionation of supernatant C-1. The 7Sγ2 globulins in solution were effectively removed by adjustment of the pH to 7.3 (Table 7) and subsequent addition of alcohol to 25%. The purity of the product was established

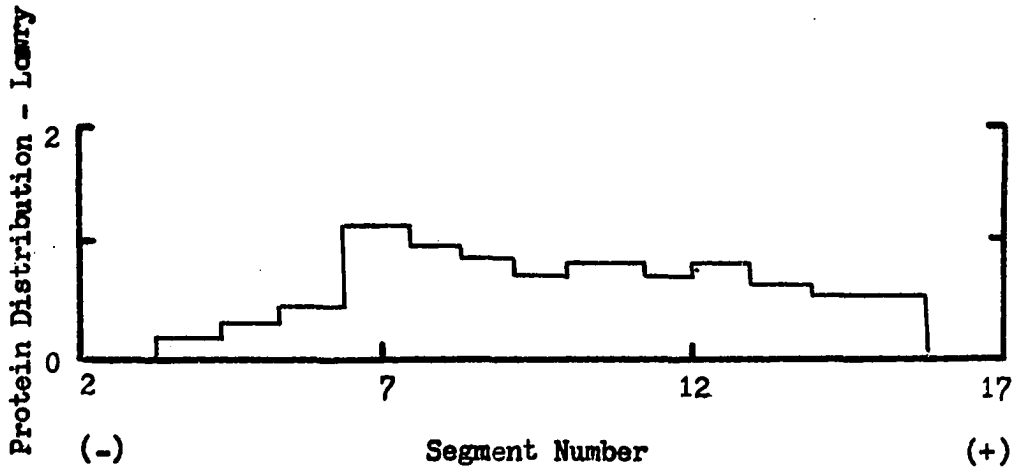

Figure 5. Distribution of ppt BB by zone electrophoresis.

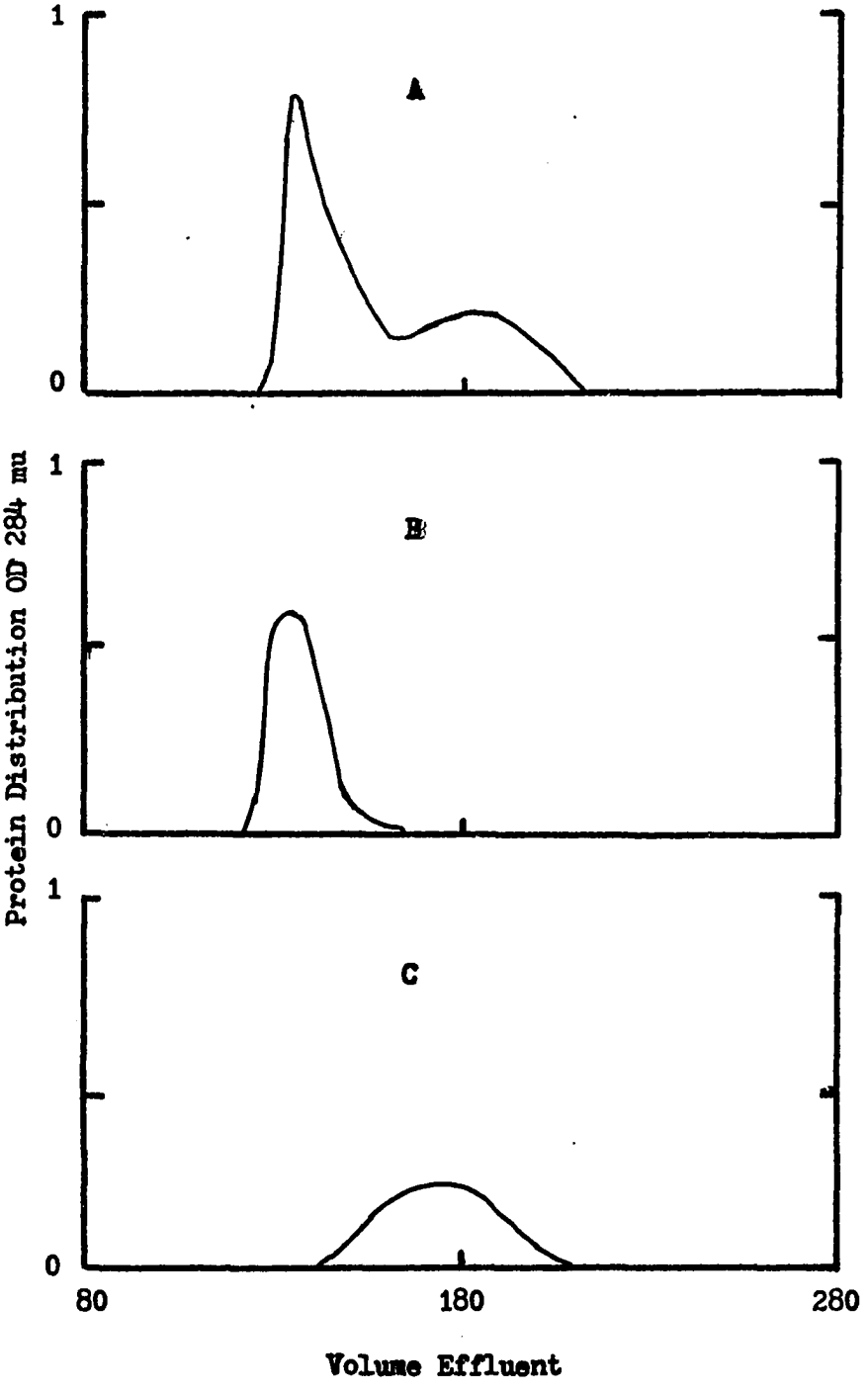

Figure 6. Gel filtration of ppt BB(A), cathodal segments from zone electrophoresis of ppt B(B) and anodal segments from zone electrophoresis(C).

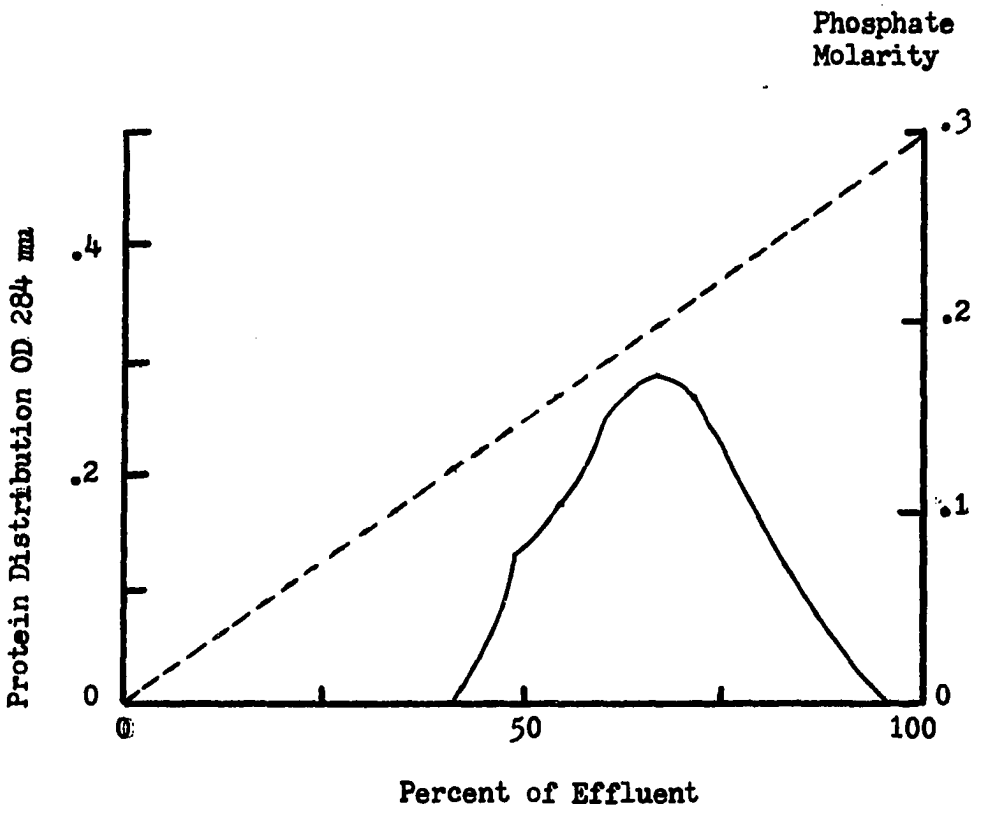

Figure 7. Ion-exchange chromatography of ppt BB.

Table 5. Effect of pH on subfractionation of supernatant B.

pH	Yield of YF globulin*
5.6	0.94
5.7	0.98
5.8	1.00
5.9	0.93
6.0	0.90

*Yield based on ratio of yield/ maximal yield.

Table 6. Effect of ionic strength on precipitation of ϒF globulins from supernatant at pH 5.8.

Ionic strength	Yield of ϒF globulins*
0.0075	0.97
0.0100	1.00
0.0125	0.98
0.0150	0.94

*Yield based on ratio of yield/ maximal yield.

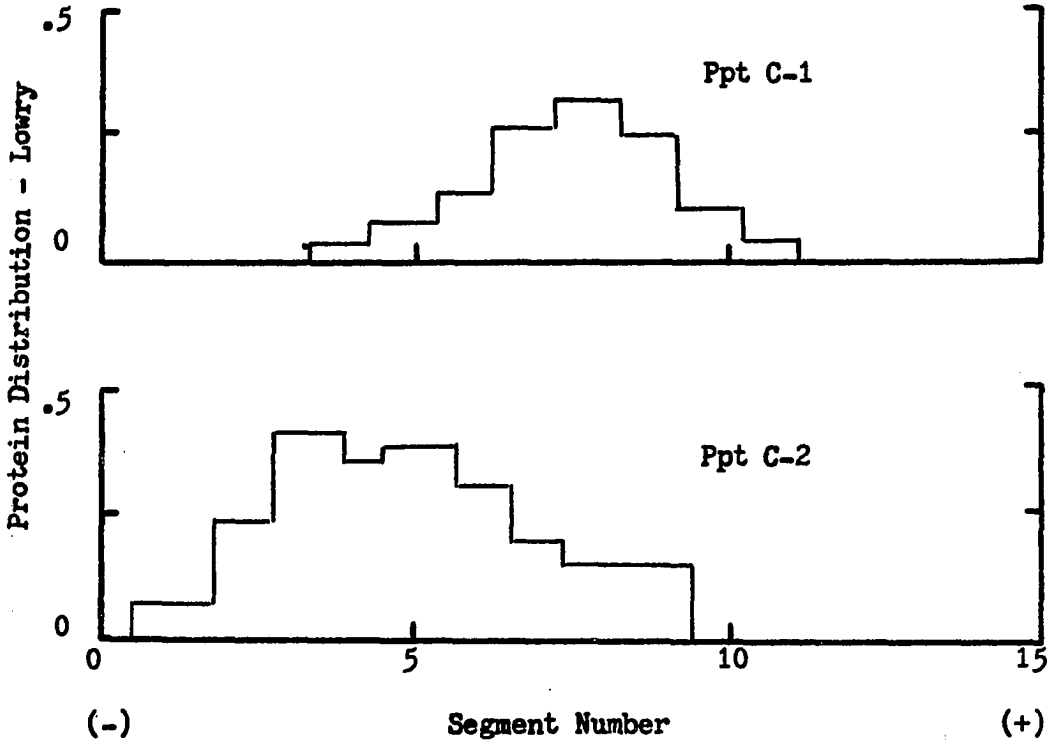

Figure 8. Distribution of ppt C-1 and ppt C-2 by zone electrophoresis.

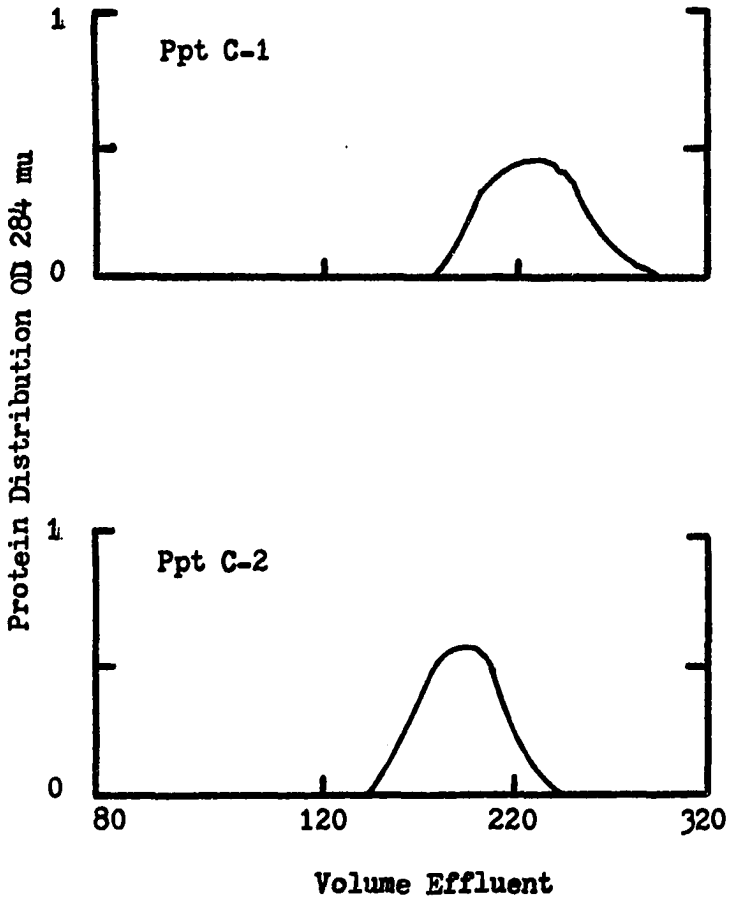

Figure 9. Distribution of ppt C-1 and ppt C-2 by Sephadex G200 chromatography.

Table 7. Effect of pH on subfraction of supernatant C-1.

pH	Yield of 7S γ2 globulins*
7.2	0.98
7.3	1.00
7.4	0.97

*Yield based on ratio of yield/ maximal yield.

by CAM electrophoresis, zone electrophoresis (Figure 8) and gel chromatography (Figure 9). Fractionation of the 7SΥ2 globulins into ΥG and ΥH globulins was not established by the methods employed. One interesting result occurred when a sample of 7SΥ2 globulin was accidently placed on the center of a starch-block and electrophoresis was allowed to run for 26 hr (Figure 10). Whether the fractions separated areΥG and ΥH globulins has not been established.

A final scheme for the fractionation of mouse serum by alcohol can be seen in Figure 11.

Evaluation of HR activity in mice. Preparations of 3.58 anti-HEA, obtained from groups of 10 mice, were used throughout this study. Forty per cent saturation by ammonium sulfate was shown to be effective in producing a homogenous Υ-globulin preparation after two fractionations. In each instance, it was necessary to demonstrate that the Υ-globulins were effectively degraded into Fab and Fc fragments and that both the mercuripapain and soluble papain treated protein reacted in the same manner. In all cases, it was found that both papain treatments degraded the protein to the same degree. The undegraded protein was effectively separated on Sephadex G100 (Figure 12).

The Fab-Fc fragments were separated by starch-block electrophoresis as illustrated in Figure 13. Only the cathodal one half of the Fab segments were used in the test. The effectiveness of the separation was demonstrated when

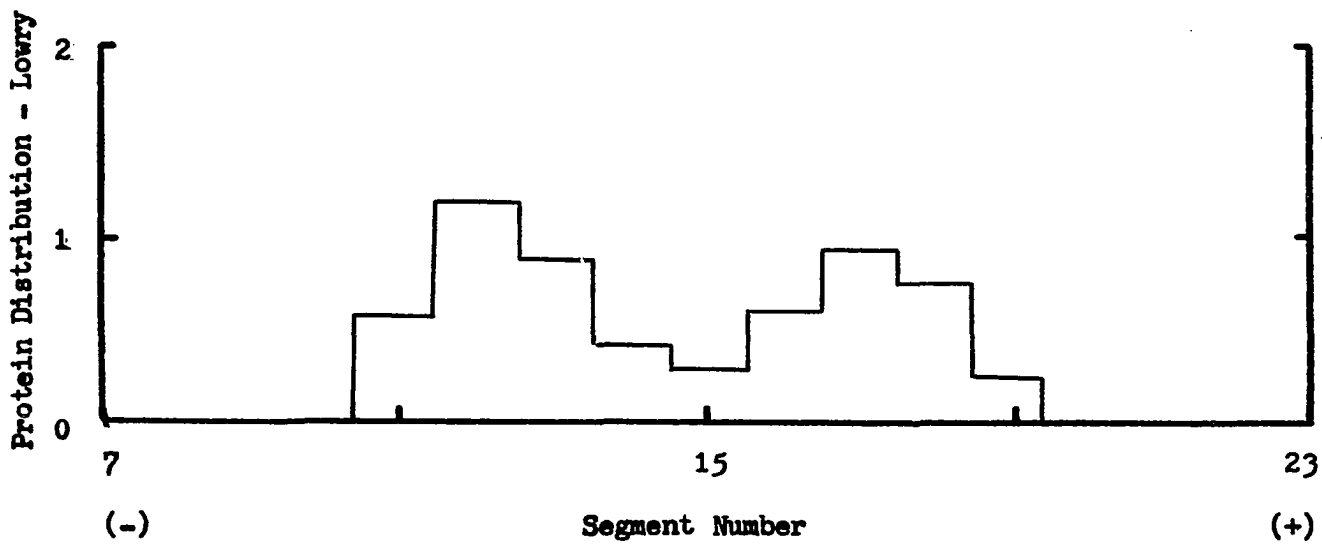

Figure 10. Separation of 7S γ2 globulins by zone electrophoresis.

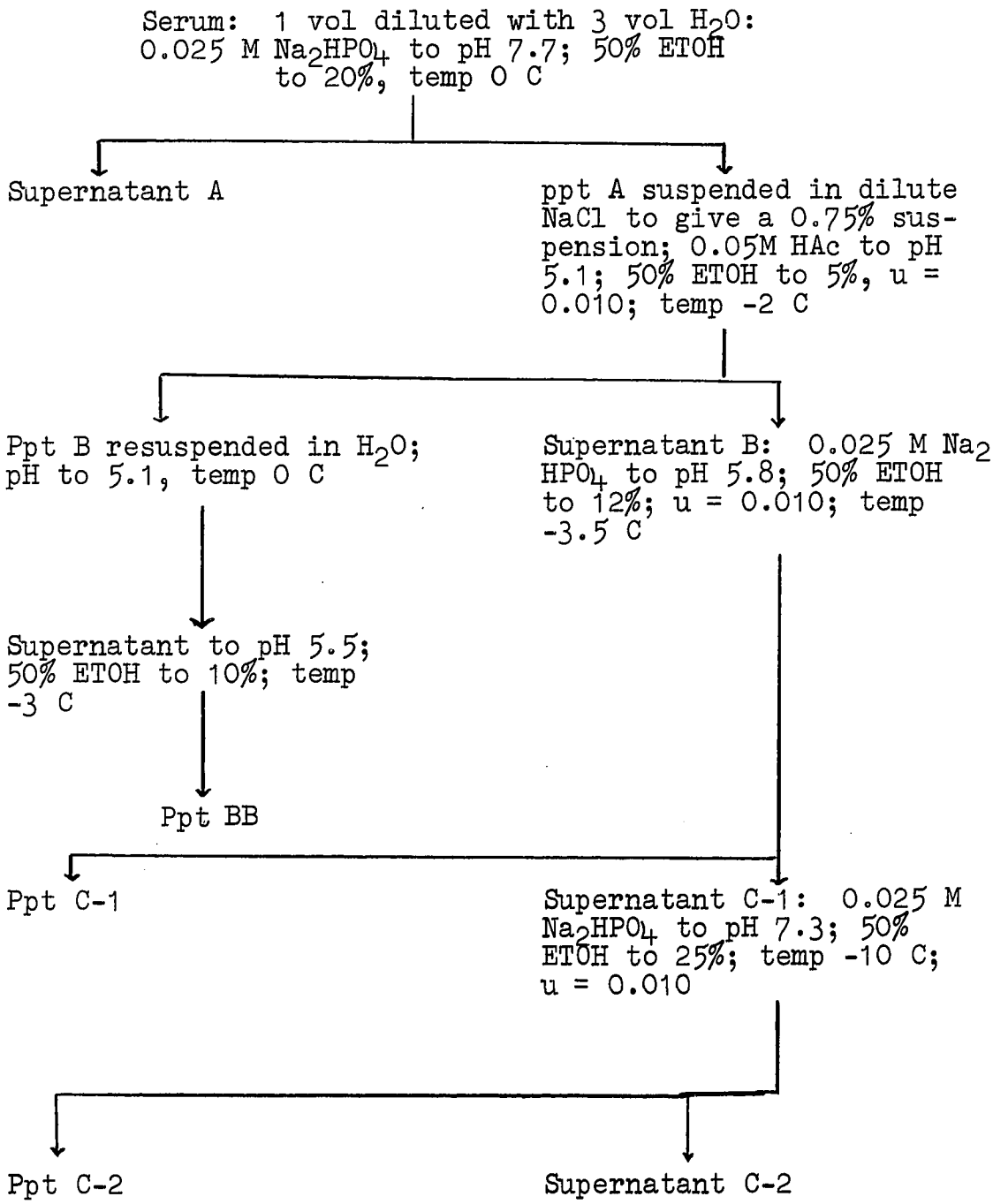

Figure 11. Final scheme for the ethanol fractionation of mouse serum.

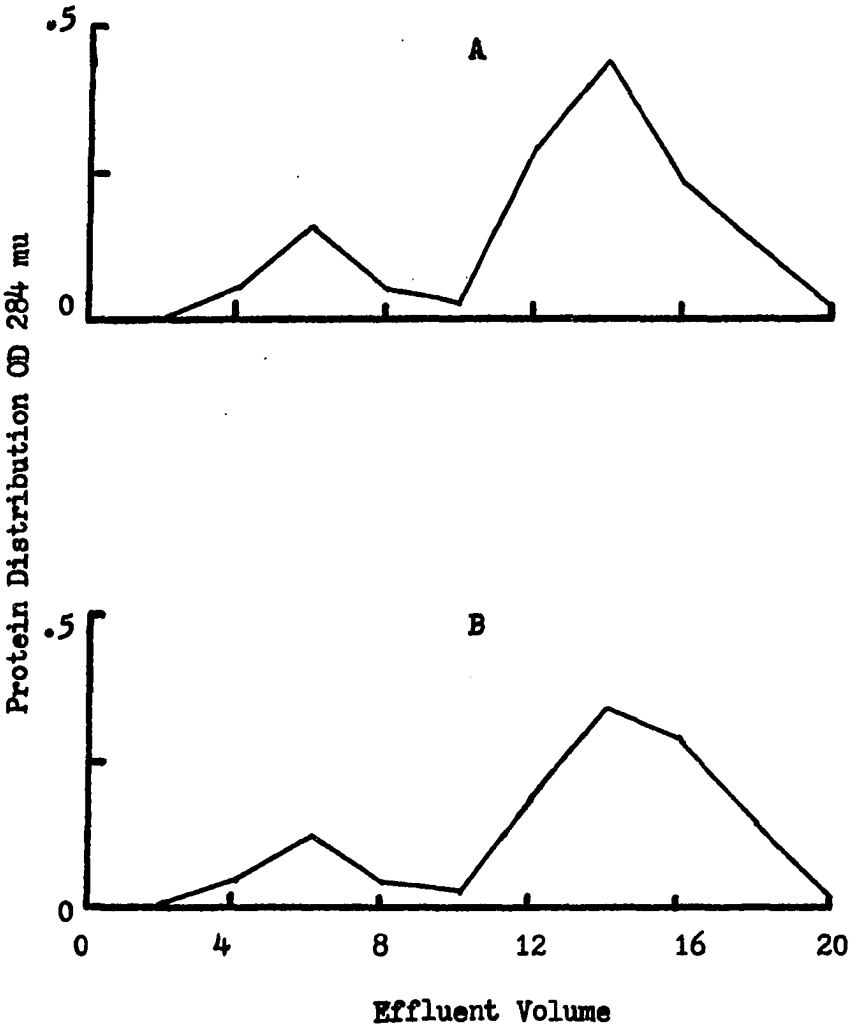

Figure 12. Separation of papain digested immunoglobulin by gel filtration. A represents soluble papain treated group 12. B represents mercuripapain treated group 16. The first peak represents undigested protein.

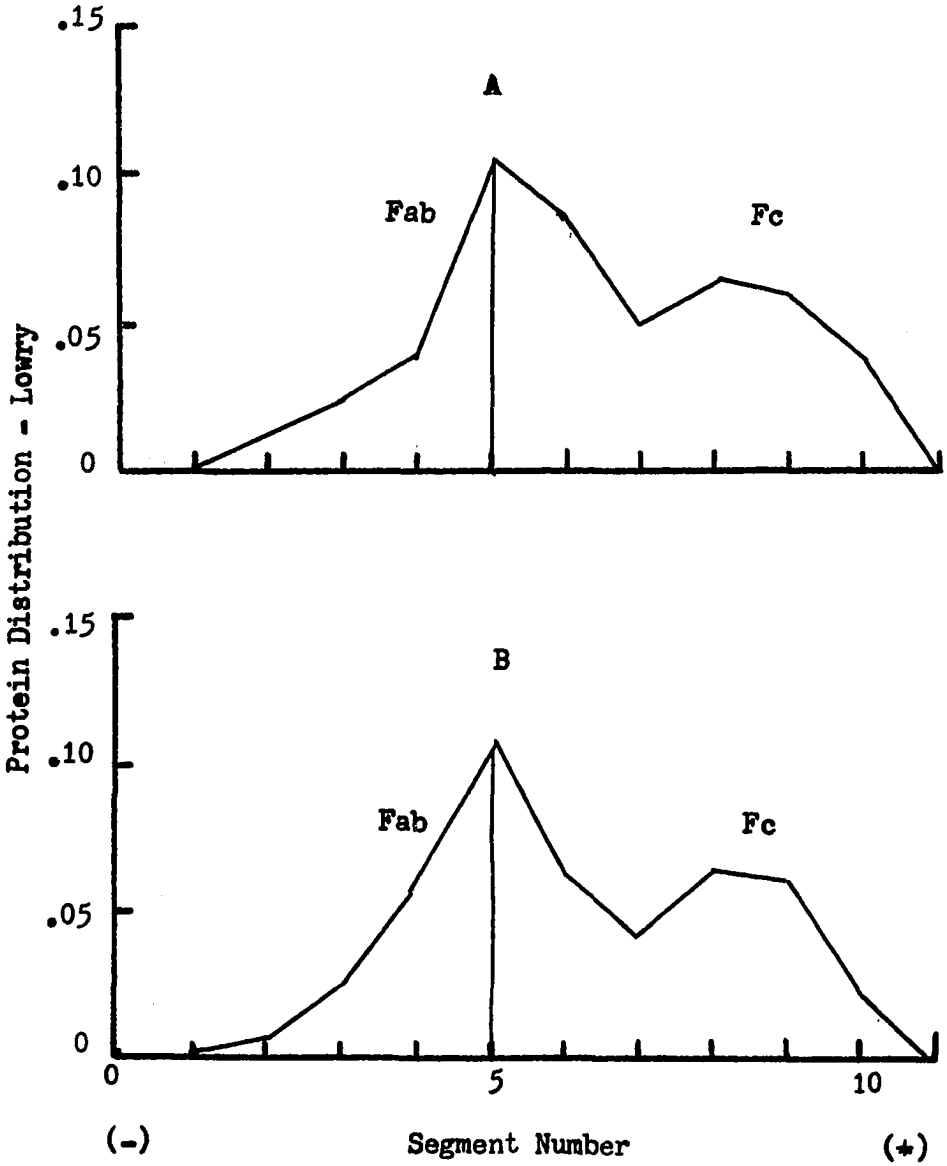

Figure 13. Separation of Fab and Fc fragments by zone electrophoresis. A represents soluble papain treated group 12. B represents mercuripapain treated group 16. Segments to the left of the vertical line were used in testing for HR activity.

the Fc segments crystallized on dialysis against 0.1 M sodium acetate while the Fab segments failed to crystallize.

In each instance, the Fab fragments failed to produce direct agglutination of human O Rh(+) erythrocytes. The Fab preparations, however, combined with antigenic receptors on the red cells, as demonstrated by an anti-globulin reaction using rabbit antiserum to native mouse ƔG-globulin (Tables 8, 9, 10).

As shown in Table 8, agglutination occurred when polled normal mouse serum was added to O Rh(+) cells sensitized with varying amounts of Fab anti-HEA preparations. The variations in the titers are due to the variance in production of anti-HEA which would be expected from a single iv injection. Similar results are seen in Table 9 where the anti-HEA was produced by a single ip injection. As shown in Table 10, higher titers were obtained from Fab preparations from mice receiving multiple injections of O Rh(+) cells.

Comparative agglutinations with varying amounts of pooled mouse serum and cells sensitized with varying amounts of Fab anti-HEA are given in Table 11. The data in this table give some indication of the quantity of HR in normal mouse serum. At the lower serum dilutions, the degree of agglutination is comparable to that of the rabbit anti-MƔG reagent.

Table 8. Agglutination of O Rh(+) cells sensitized with Fab anti-HEA preparations from groups of 10 Swiss mice.

Group no	Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]					
			4	16	64	256	1024	4096
1	ws	0	0	0	0	0	0	0
2	ws	0	3+	1+	0	0	0	0
3	ws	0	4+	4+	3+	1+	0	0
4	ws	0	4+	3+	2+	±	0	0
5	ws	0	2+	1+	±	0	0	0
6	ws	0	1+	0	0	0	0	0
	PBS	0	0	0	0	0	0	0
	MTG Ab	0	4+	4+	3+	3+	1+	0

[a]Indicates reagent used, ws = whole serum of same strain; PBS = phosphate buffered saline; MTG Ab = antibody produced against mouse globulin.

[b]Indicates phosphate buffered saline.

[c]Indicates anti-HEA produced by a single iv injection of 1.8×10^8 human O Rh(+) cells.

Table 9. Agglutination of O Rh(+) cells sensitized with Fab anti-HEA preparations from groups of 10 Swiss mice.

Group no	Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]					
			4	16	64	256	1024	4096
7	ws	0	0	0	0	0	0	0
8	ws	0	4+	2+	±	0	0	0
9	ws	0	4+	4+	3+	1+	0	0
10	ws	0	4+	3+	2+	±	0	0
11	ws	0	3+	2+	±	0	0	0
12	ws	0	2+	±	0	0	0	0
	PBS	0	0	0	0	0	0	0
	MYG Ab	0	4+	4+	4+	3+	0	0

[a]Indicates reagent used, ws = whole mouse serum of same strain; PBS = phosphate buffered saline; MYG Ab = antibody produced against mouse globulin.

[b]Indicates phosphate buffered saline.

[c]Indicates anti-HEA produced by a single ip injection of 1.8×10^8 human O Rh(+) cells.

Table 10. Agglutination of O Rh(+) cells sensitized with Fab anti-HEA preparations from groups of 10 mice.

Group no	Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]					
			4	16	64	256	1024	4096
13	ws	0	4+	4+	3+	3+	2+	0
14	ws	0	4+	4+	3+	2+	±	0
15	ws	0	4+	4+	4+	2+	1+	0
16	ws	0	4+	4+	3+	2+	1+	0
17	ws	0	4+	4+	3+	1+	±	0
18	ws	0	4+	4+	3+	2+	1+	0
19	ws	0	4+	4+	2+	1+	±	0
	PBS	0	0	0	0	0	0	0
	MYG Ab	0	4+	4+	4+	3+	2+	1+

[a]Indicates reagent used, ws = whole mouse serum of same strain; PBS = phosphate buffered saline, MYG Ab = antibody produced against mouse globulin.

[b]Indicates phosphate buffered saline.

[c]Indicates anti-HEA produced by multiple injections of 1.8×10^8 human O Rh(+) cells.

Table 11. Agglutination of sensitized O Rh(+) cells by pooled normal mouse serum.

Dilutions of normal serum	PBS[b]	Reciprocal dilutions of Fab anti-HEA[a]				
		4	16	64	256	1024
1:2	0	4+	4+	4+	3+	1+
1:8	0	4+	4+	3+	2+	1+
1:32	0	4+	3+	3+	2+	±
1:128	0	0	0	0	0	0
1:512	0	0	0	0	0	0
PBS	0	0	0	0	0	0
MγG Ab[c]	0	4+	4+	4+	3+	3+

[a]Indicates Fab anti-HEA from group no 16.

[b]Indicates phosphate buffered saline.

[c]Indicates antibody against mouse globulin.

The result of agglutination tests with the fractions obtained by ethanol fractionation are shown in Table 12. The HR activity was found in the 7SY2 fraction and to a lesser degree in the YM fraction. The activity of the YM fraction was confirmed by the use of mercaptoethanol on the YM before addition of cells (Table 13).

The effect of the addition of Fab fragments of normal mouse YG-globulin on the agglutination of sensitized cells of polled mouse YG-globulin is illustrated in Table 14. The inhibitory power of the Fab fragments appears to be dependent upon the relative amount of HR in the mouse.

The effect of cross agglutination titers between different strains of mouse Fab anti-HEA can be seen in Tables 15, 16, 17. In all cases, the titers were positive to varying degrees and lower than the titer of the same strain tested against its on strain. Generally, the titer dropped from 2 to 4 tube dilutions.

Table 12. Agglutination of sensitized O Rh(+) cells by fractions of normal mouse serum.

Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]					
		2	8	32	128	512	2048
γF	0	0	0	0	0	0	0
7Sγ2	0	4+	4+	4+	4+	3+	1+
γA	0	0	0	0	0	0	0
γM	0	4+	3+	2+	±	0	0
PBS	0	0	0	0	0	0	0
MγG Ab	0	4+	4+	4+	4+	3+	3+

[a]Indicates fractions of immunoglobulins produced by ethanol fractionation; PBS = phosphate buffered saline; MγG = antibody produced against mouse globulins.

[b]Indicates phosphate buffered saline.

[c]Indicates anti-HEA from group 12.

Table 13. Effect of 2-mercaptoethanol on the agglutination of sensitized cells with normal ϒM mouse globulin.[c]

Mercapto-ethanol[a]	PBS[b]	Reciprocal dilutions of normal ϒM globulin			
		2	8	32	128
0.05 M	0	2+	±	0	0
0.10 M	0	0	0	0	0
0.15 M	0	0	0	0	0
PBS	0	4+	4+	2+	±

[a]Indicates the final concentration of reducing agent added to serum fraction.

[b]Indicates phosphate buffered saline.

[c]Indicates that the cells were sensitized according to methods; Fab anti-HEA from group no 18.

Table 14. Inhibition of homoreactant activity by fragments of normal mouse YG-globulin.

Serum dilution	Unsensitized cells	Inhibitor concentration 1:4	1:16	1:64	1:256	PBS
1:4	0	0	1	4	4	4
1:16	0	0	1	2	3	3
1:64	0	0	0	1	2	3
1:256	0	0	0	0	0	0
PBS[a]	0	0				

[a]Indicates phosphate buffered saline.

Table 15. Agglutination of human O Rh(+) cells sensitized with Fab anti-HEA preparations from Swiss mouse serum.

Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]				
		4	16	64	256	1024
Swiss serum	0	4+	4+	4+	2+	1+
C57 BL/6J serum	0	3+	2+	1+	0	0
CBA/J serum	0	3+	2+	2+	1+	0
PBS	0	0	0	0	0	0
anti-MγG	0	4+	4+	4+	3+	1+

[a]Indicates whole serum from strain used.

[b]Indicates phosphate buffered saline.

[c]Indicates Fab anti-HEA from Swiss mice, group 15.

Table 16. Agglutination of human O Rh(+) cells sensitized with Fab anti-HEA preparations from C57 BL/6J mouse serum.

Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]				
		4	16	64	256	1024
C57 BL/6J serum	0	4+	4+	3+	3+	1+
Swiss serum	0	3+	2+	2+	1+	0
CBA/J serum	0	3+	2+	1+	0	0
PBS	0	0	0	0	0	0
Anti-MγG	0	4+	4+	4+	2+	1+

[a]Indicates whole serum from strain used.

[b]Indicates phosphate buffered saline.

[c]Indicates Fab anti-HEA from C57 BL/6J group 17.

Table 17. Agglutination of human O Rh(+) cells sensitized with Fab anti-HEA preparations from CBA/J mouse serum.

Reagent[a]	PBS[b]	Reciprocal dilutions of Fab anti-HEA[c]				
		4	16	64	256	1024
BBA/J serum	0	4+	4+	2+	1+	0
Swiss serum	0	3+	2+	2+	1+	0
C57 BL/6J	0	3+	2+	1+	0	0
PBS	0	0	0	0	0	0
Anti-MγG	0	4+	4+	4+	3+	1+

[a]Indicates whole serum from strain used.

[b]Indicates phosphate buffered saline.

[c]Indicates Fab anti-HEA from CBA/J group 19.

CHAPTER IV

DISCUSSION

Ethanol fractionation of mouse serum. The results of these experiments definitely show that mouse serum could effectively be separated into its immunoglobulin components by ethanol fractionation. The technique was effective in fractionating mouse serum into three categories; γA-γM globulins, γF globulins, and 7Sγ2 globulins. The method was substantially faster and easier to perform than the method of Fahey et al. (10) or the method of Murgita and Vas (27). It also gave purer products than are obtainable by salting-out procedures (13).

The secondary fraction of mouse serum was more closely related to human serum rather than rat serum, as expected (3, 28). The results of this step were somewhat surprising.

The γA and γM globulin combination of precipitate BB was separated into its respective components by gel filtration. The γM globulin, being a 19S protein (10), rapidly proceeded the γA globulin through the column. Due to the 13 S component of γA (8), total separation was not

achieved. However, recycling gel chromatography or chromatography through Sepharose 4B would give total separation (26).

Treatment of the γA-γM combination by zone electrophoresis was also effective, but not to the degree of gel chromatography. This was due to the overlapping of the mobilities of the two globulins (10). The inability to separate the two components by ion-exchange chromatography was evidently due to the charges on the molecules. From zone electrophoresis, it was shown that both molecules contained similar total electrical charges and therefore, would be very difficult to separate by ion-exchange chromatography.

There are several explanations for the low yield of γA and γM in the purified precipitate (ppt BB). The stringent acidic conditions of the fractionation procedure combined with the action of alcohol on proteins may cause a denaturation of the molecules. This would seem probable since both γM and γA globulins are polymers of subunits and are weakly stable at isotonic conditions (8). A second possibility is that one form of the γM globulin (19SγM as aversed to γMS-N) is lost in the fractionation procedure (8). A third possibility exists that the majority of the γA and γM globulins are precipitated. The amounts of γA and γM globulins that Fahey et al. (10) obtained in their fractionation methods were based on different strains of

mice than were used in this study. Fahey et al. (10) also reported different immunoglobulin levels for each strain used. Therefore, it is possible that most of the globulins were precipitated in this study.

The ability to separate γF globulins from the 7S globulins was an important step in the procedure. This seemed particularly important since the biological function(s) of the γF globulin is not known and pure preparations were needed before this question can be answered.

The 7Sγ2 globulins left in solution after fractionation of γF were effectively precipitated. The subfractionation of the 7Sγ2 globulins into its γG and γH components should be studied. The two components, originally discovered with myeloma proteins, are of the same molecular weight. However, there is a possibility that they may be separable by electrophoresis. Each myeloma protein of the γG and γH class seems to have a separate electrophoretic mobility (11). The testing of each γG and γH myeloma protein against protein segments obtained from zone electrophoresis (Figure 10) may be fruitful in separation of the 7Sγ2 components (11).

The present work was undertaken as a study of the normal serum immunoglobulins of mice, but it becomes a matter of importance to distinguish the anomolous immunoglobulins. It was not possible in this study to determine

such proteins, but they are generally found in extremely low amounts in normal animals (10).

The results obtained from zone electrophoresis and gel filtration agree with the criteria for purity obtained by Fahey et al. (10) giving crediance to the results of the fractionation.

Detection of homoreactant in mouse serum. The experiments reported in this paper demonstrated the presence in the serum of all normal mice tested of an anti-globulin factor, homoreactant, which specifically reacts with the Fab fragments of papain digested mouse γG-globulin. This factor was detected by the agglutination of erythrocytes sensitized with the univalent fragments of mouse anti-HEA antibody with normal mouse serum.

Inhibition of this factor by the Fab fragments of normal mouse γG-globulin, but not by the intact γG-globulin molecule indicated that this factor was directed toward a hidden antigenic site on the Fab fragment (18).

The results of tests with ethanol fractions of mouse serum indicated that there are two types of homoreactant present in mice; a 7Sγ2 globulin and a γM globulin form. It was not feasible to tell if the activity of homoreactant resided in the γG or γH subfraction of 7Sγ2 globulin. It was not, however, the Xγ component of normal mouse serum since this component would be precipitated with the γA and γM globulins during ethanol fractionation (10).

Since the results of this series of experiments agreed with the results obtained from rabbits (18), it can be assumed that the γM globulin form of homoreactant was also directed to determinants which are otherwise buried on the intact γG-globulin molecule and exposed by digestion with papain.

It is known that the light chains of γM and 7Sγ2 globulins are alike, while the heavy chains are different (37). It is also known that after papain digestion, the invariable region of the heavy chain is removed, leaving the variable region of the heavy chain adjacent to the invariable region of the light chain (37). Therefore, there are two possibilities for the site of homoreactant. One, the process of papain digestion exposed a site on the light chain invariable region through a denaturation process. This is improbable since papain digestion has no known denaturation affect on light chains. Secondly, the site of action of the Fab fragment is located on the variable region of the chain. This would seem likely since the internal portion of the heavy chain is now exposed (37). If this were true, it would give an invariable region in the variable region of the heavy chain.

Many immunologists consider the demonstration of an amnestic-like response following immunization as an important step in showing that a naturally occurring factor is an antibody. The results of such experiments would be inconclusive since an increase in titer might represent the

production of new antibody rather than an increase in titer of the natural occurring antibody (19).

The relationship between anti-antibody and homoreactant is not certain. However, homoreactant is easily neutralized by the Fab fragments of normal mouse ϒG-globulin, whereas, anti-antibody of the rabbit is not neutralized (18). Furthermore, anti-antibody is associated with sites revealed by the union of antigen-antibody, while homoreactant is not.

Homoreactant in the mouse would also differ from rheumatoid-like factors formed in experimental animals, since rheumatoid factors are mercaptoethanol sensitive (18).

The possibility that homoreactant in the mouse is related to allotypic antibodies should be investigated.

CHAPTER V

SUMMARY AND CONCLUSIONS

Ethanol fractionation. A method for the ethanol fractionation of normal mouse serum has been developed. The method was shown to be rapid and simple to perform.

The method was shown to be effective in purification of 7SΥ2, ΥF, and ΥM-ΥA globulins. Gel filtration and to a lesser degree, zone electrophoresis, proved to be effective in separating the ΥM- ΥA globulin combination into their respective components.

The parameters chosen to assess the immunological purity of the fractions included zone electrophoresis, gel filtration and cellulose-acetate electrophoresis. In all cases, the fractionation products agreed chemically with the results obtained by other workers.

Homoreactant. A study to investigate the presence of homoreactant in mice was undertaken. Mice were injected with human O Rh(+) erythrocytes. The serum from the mice was digested with papain and the 3.5S univalent (Fab) fragments separated from the Fc fragments. The reaction was demonstrated by agglutination of human O Rh(+) erythrocytes

sensitized with the Fab fragments when treated with normal mouse serum.

The activity of the serum factor was inhibited by the 3.5S univalent fragments of normal mouse ϒG-globulin. The results suggested that there are two types of homo-reactant in mice; a 7Sϒ2 globulin form and a ϒM globulin form. The latter was shown to be mercaptoethanol suceptible. It was also demonstrated that there was cross-reactivity between strains of mice.

The data suggests that papain digestion reveals a hidden antigenic site on mouse ϒG-globulin.

BIBLIOGRAPHY

1. Baunstark, J. S., R. J. Laffin and M. A. Bardarid. 1964. A preparative method for the separation of 7S gamma globulin from human serum. Arch. Biochem. Biophys. 108:514-522.

2. Cohn, E. J., L. E. Strong, W. L. Hughs, Jr., D. J. Mulford, J. M. Ashworth, M. Melin, and H. L. Taylor. 1946. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the proteins and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc. 68:459-475.

3. Deutsch, H. L., L. J. Gostling, R. A. Alberty, and J. W. Williams, 1946. Biophysical studies of blood plasma proteins. III. Recovery of γ-globulins from human blood protein mixtures. J. Biol. Chem. 164:109-118.

4. Deutsch, H. F., R. A. Alberty, and L. A. Gostling. 1946. Biophysical studies of blood plasma proteins. III. Recovery of γ-globulin from human blood protein mixtures. J. Biol. Chem. 164:109-118.

5. Deutsch, H. F., R. A. Alberty, and L. A. Gostling. 1946. Biophysical studies of blood plasma proteins. II. Separation and purification of a new globulin from normal human plasma. J. Biol. Chem. 165:21-35.

6. Dray, S. and G. O. Young. 1958. Differences in the antigenic components of sera of individual rabbits as shown by induced isopreciptins. J. Immunol. 81:142-149.

7. Dray, S., R. Lieberman, and H. Hoffman. 1963. Two immune γ-globulin allotypic specificities identified by ascitic fluid isoprecitins and determined by allelic genes. Proc. Soc. Exp. Biol. Med. 113:509-513.

8. Fahey, J. L. 1963. Heterogeneity of gamma globulins. Adv. Immunol. 2:41-67.

9. Fahey, J. L. 1967. Chromatographic separation of immunoglobulins, p. 321-332. In C. Williams and M. Chase (ed.) Methods in immunology and immunochemistry. Academic Press, New York.

10. Fahey, J. L., J. Wunderlich, and R. Mischell. The immunoglobulins of mice. I. Four major classes of immunoglobulins; 7SΥ2-, 7SΥ1-, Υ1A (B_2A)-, and 18Υ1M- globulins. J. Expt. Med. 120:233-242.

11. Ibid. 1964. The immunoglobulins of mice. II. Two subclasses of mouse 7SΥ2-globulins; Υ2a- and Υ2b- globulins. J. Expt. Med. 120:243-251.

12. Kekwick, R. A. 1940. The serum proteins in multiple myelomas. Biochem. J. 34:1248-1257.

13. Kendall, F. E. 1938. Immunochemical determination of serum proteins. Cold Spring Harbor Symp. Quant. Biol. 6:376-384.

14. Killander, J. 1964. Separation of human heme- and hemoglobulin binding plasma proteins, ceruloplasma and albumin by gel filtration. Biochem. Biophys. Acta. 93:1-14.

15. Kimmel, J. R. and E. L. Smith. 1954. Crystalline papain. I. Preparation, specificity and activation. J. Biol. Chem. 207:515-531.

16. Kormeier, L. C., J. T. Ing, and W. J. Mandy. 1968. Specificity of antiglobulin factors in normal human serum reacting with enzyme digested ΥG-globulin. J. Immunol. 100:612-621.

17. Lowry, O. H., N. J. Rosebrough, H. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

18. Mandy, W. J., H. H. Fudenberg, and F. B. Lewis. 1965. A new serum factor in normal rabbits. I. Identification and characterization. J. Immunol. 95:501-509.

19. Mandy, W. J. 1966. A new serum factor in normal rabbits. II. Reaction with buried antigenic determinants revealed after papain digestion. J. Immunol. 97:876-884.

20. Mandy, W. J. 1967. A new serum factor in normal rabbits. III. Specificity for antigenic determinants uncovered by papain or pepsin digestion. J. Immunol. 99:815-824.

21. Mandy, W. J., M. E. Woolsey, and F. B. Lewis. 1968. A new serum factor in normal rabbits. IV. Occurrence in neonatal rabbit sera. J. Immunol. 100:15-23.

22. Mandy, W. J. and L. C. Kormeier. 1966. Homoreactant: a natural occurring autoantibody in rabbits. Science 154:651-652.

23. Merryman, C., B. Frangione, E. C. Franklin, and B. Benacerraf. 1967. Peptide differences in purified mouse antibodies. Immunochem. 4:31-36.

24. Milgrom, F. and S. Dubiski. 1957. Antigencity of antibodies of the same species. Nature 179:1351-1352.

25. Milgrom, F. and F. Witeboky. 1960. Rabbit antibodies against γ-globulins resembling the rheumatoid factor. Fed. Proc. 19:197.

26. Mischell, R. Personal communication.

27. Murgita, R. A. and S. A. Vas. 1970. Isoelectric separation of mouse immunoglobulins. J. Immunol. 104:514-517.

28. Nichol, J. C. and H. F. Deutsch. 1948. Biophysical studies of blood plasma proteins. VII. Separation of γ-globulin from the sera of various animals. J. A. Cehm. Soc. 70:80-83.

29. Osterland, C. K. 1968. Zone electrophoresis on powder blocks, p. 57-67. In C. Williams and M. Chase (ed.) Methods in immunology and immunochemistry. Vol. II. Academic Press, New York.

30. Ibid. 1968. Powder block electrophoresis, p. 409-414. In C. Williams and M. Chase (ed.) Methods in immunology and immunochemistry. Vol. II. Academic Press, New York.

31. Petermann, M. L. 1942. The activation of beef papain on beef serum pseudoglobulin and on diptheria antitoxin. J. Biol. Chem. 144:607-616.

32. Peterson, E. A. and A. A. Sorber. 1956. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. J. Am. Chem. Soc. 78:751-763.

33. Porath, J. and N. Ui. 1964. Chemical studies on immunoglobulins. I. A new preparative procedure for γ-globulins employing glycine-rich solvent systems. Biochem. Biophys. Acta. 90:324-333.

34. Porter, R. R. 1958. Separation and isolation of fractions of rabbit gamma globulins containing antibody and antigenic combining sites. Nature 182: 670-671.

35. Ibid. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. 73:119-126.

36. Potter, M. and R. L. Liebermann. 1967. Genetics of immunoglobulins in the mouse. Adv. Immun. 7:91-145.

37. Putman, F. W. 1969. Immunoglobulin structure: variability and homology. Science 163:633-644.

38. Steiner, L. A. and S. Lowey. 1966. Optical rotatory dispersion studies on rabbit γG-globulin and its papain fragments. J. Biol. Chem. 241:231-240.

39. Talal, N., G. Hermann, C. de Vaux St. Cyr, and P. Graber. 1963. Immunoelectrophoretic study of mouse serum and urinary esterases. J. Immunol. 90:246-256.

40. Vareman, J. P., J. F. Heremans, and C. Vareman. 1963. Studies on the immune globulins of human serum. I. A method for simultaneous isolation of the three immune globulins (γSS, γ1M, and γ1A) from individual small serum samples. J. Immunol. 90: 64-72.

41. Vareman, J., H. Fudenberg, C. Vareman, and W. Mandy. 1965. On the significance of the heterogenecity in molecular size of human serum γA-globulins. Immunochem. 2:263-272.

42. Waller, M. and K. Blaylock. 1966. Further studies on the anti-globulin factors in human serum to the pepsin digested fragments of RI-anti-Rh antibody. J. Immunol. 97:438-443.