JCI The Journal of Clinical Investigation

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J Clin Invest. 1957;36(3):456-467. https://doi.org/10.1172/JCI103443.

Research Article

PHYSICO-CHEMICAL AND IMMUNOLOGIC STUDIES ON MACROGLOBULINS [1]

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(Submitted for publication October 8, 1956; accepted November 29, 1956)

Normal sera are resolved in the ultracentrifuge into two major peaks with sedimentation constants of the order of 4.5 and 6.5 S [2] and a minor peak of heavy materials, amounting to less than 3 per cent of the total proteins, with a sedimentation constant of about 20 S.

In 1944, Waldenström (1) reported the presence of a large amount of fast sedimenting moieties (19 to 20 S) in sera of some patients with marked hyperglobulinemia. This finding prompted him to assume that these serum components were of high molecular weight and he named them macroglobulins. He also reported that these serum components could be precipitated out on dilution of the serum with 16 volumes of distilled water.

In recent years a number of workers have reported the presence of components in pathological sera with properties similar to those assigned by Waldenström to macroglobulins (2–10). However, in a number of cases the macroglobulins detected by the precipitation test with distilled water had sedimentation constants as low as 12 S and as high as 30 S. On the basis of electrophoretic mobilities, macroglobulins have been reported to occur either in the β or γ-globulin regions (2, 3, 5, 6, 8–11).

Vogler, Oberhänsli, and Kofler (12) found that the diffusion coefficient for some macroglobulins was 1.2×10^{-7} cm^2. per sec. in contrast to the accepted value for normal γ-globulins of about 3.8×10^{-7} cm^2. per sec. This finding was considered further evidence for the high molecular weight of macroglobulins.

In 1952, Derrien (11) showed that macroglobulins from different pathological sera which possessed similar electrophoretic and ultracentrifugal characteristics nevertheless could be differentiated by their solubility properties. In addition he showed that macroglobulins, apparently homogeneous by electrophoresis, were markedly heterogeneous on the basis of solubility properties.

Recently a number of authors have reported immunologic studies on macroglobulins. Habich (3) concluded that the macroglobulins in some sera did not possess any distinct antigenic groups with respect to normal sera. On the other hand, the macroglobulins of other sera exhibited specific antigenicity. On the basis of immunologic tests with these latter sera, he suggested that some macroglobulins contained group-specific as well as individually specific antigens. Grümer and Klaus (7), using rabbit antiserum to macroglobulin, reported that the macroglobulin serum contained specific antigens not found in normal human serum. Di Guglielmo and Antoninix (2) investigated the immunologic properties of macroglobulins using the anaphylaxis reaction as the criterion for identity of antigens and suggested that macroglobulins possessed individually specific antigenic groups not found in normal human serum.

The amino-acid composition of the macroglobulins has recently been investigated (5–7). The β- and γ-macroglobulins appeared to differ only slightly in their amino-acid content from normal β- and γ-globulins.

During the past two years, we have investigated the sera from four patients with macroglobulinemia. Their case histories have been reported elsewhere (13). The physico-chemical and immunologic results are reported here.

[1] Supported by a grant from the Department of National Health and Welfare, Canada. We are also indebted to Charles E. Frosst Co., F. W. Horner Co., Montreal and Eli Lilly Co., Indianapolis, for assistance. Presented in part at the meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N. J., April, 1956.

[2] S refers to Svedberg units.

METHODS

Isolation of macroglobulins

Each of the sera was diluted 16-fold with distilled water. The macroglobulins [3] which precipitated out were centrifuged and the precipitates were washed 3 times with cold distilled water. The precipitates were then dissolved in saline (0.9 per cent NaCl). In attempts at further purification, the macroglobulins of two sera were reprecipitated twice from the saline solution by addition of distilled water. In view of the close similarity of the ultracentrifugal and electrophoretic patterns after the first and third precipitations, the macroglobulins of the other two sera were precipitated only once, thus limiting the inherent losses associated with successive reprecipitations. The macroglobulins in saline solution were Seitz-filtered into sterile vials and stored at 4° C. After precipitation of the macroglobulins, the supernatants were concentrated to the original volumes of the sera by pervaporation in Visking tubing under sterile conditions. The whole sera, the precipitated macroglobulins and the supernatants were compared by electrophoresis, ultracentrifugation and immunologic methods.

Electrophoresis

1) *Free electrophoresis.* All samples were examined in a Spinco model H Tiselius apparatus at 0.8° C, using veronal buffer at pH 8.6 and ionic strength 0.1. The solutions were dialyzed through Visking tubing against the buffer for a period of 24 to 36 hours prior to electrophoretic analyses. The protein concentration of each sample was about 1 per cent as determined refractometrically (14). The macroglobulin fraction of one serum (A.B.) was analyzed in acetate buffer at pH 3.65 and 4.8, phosphate buffer at 6.5, 7.0, and 7.7 and veronal buffer at pH 8.6. The ionic strength of all buffers was 0.1.

2) *Paper electrophoresis.* All samples were investigated by paper electrophoresis, according to a procedure described previously (15). Veronal buffer (pH 8.6, ionic strength 0.1) was used in this study. After electrophoresis the papers were stained for proteins and carbohydrates with Amido black 10B (16) and the periodic acid Schiff reagent (17), respectively.

3) *Starch electrophoresis.* One serum (A.B.) was separated into its electrophoretically distinct components using starch electrophoresis. The method is fully described elsewhere (18). The starch block was divided into segments according to the protein distribution curve as shown in Figure 5. The serum protein fractions were eluted from the starch block and examined in the ultracentrifuge and by paper electrophoresis.

As shown in Figure 5, the γ_2-globulins were divided into four sub-fractions in an attempt to delimit more precisely the locale of the macroglobulins.

[3] In this paper the term macroglobulins refers to serum components precipitated by 16-fold dilution of pathological serum with distilled water. This treatment did not cause precipitation of the generally found fast sedimenting component of normal serum ($S \simeq 20$).

Ultracentrifugation

The Spinco model E optical ultracentrifuge was used to determine the sedimentation constants of the proteins in different samples. The solutions were made up in or were diluted with saline. The average temperature during centrifugation was 19 to 20° C and the rotor speed was 59,780 r.p.m. The sedimentation constants were not recalculated for standard conditions. The effect of protein concentration on sedimentation constants was not investigated in view of the apparent complexity of these materials.

Immunologic methods

Albino rabbits of both sexes (4 to 8 kg. body weight) were immunized with the following antigens: 1) the macroglobulin fraction of serum A.B. (1.5 per cent in saline), referred to hereafter as M, 2) undiluted pooled normal human serum referred to as NHS, 3) Squibb gamma globulins [4] (3.5 per cent in saline) referred to as GG. One-ml. aliquots of each antigen solution were injected intravenously into two rabbits three times a week (on 3 successive days) for a period of four to five weeks. The animals were bled 6 to 9 days after the last injection. The antisera to each antigen were pooled, Seitz-filtered into sterile bottles and stored at 4° C. The antisera were tested for presence of antibody by ring test.

To determine the degree of antigenic similarity between M and GG, the following experiment was carried out (experiment A). In a series of 19 tubes, 1-ml. aliquots of the antiserum to M, diluted fourfold, were mixed with equal volumes of a solution of GG in halving dilutions. The maximum concentration of the GG solution used was 9 per cent. Incubation of the tubes for 2 hours at 37° C followed by incubation for 48 hours at 4° C resulted in the formation of visible precipitates. The tubes were then centrifuged and aliquots of the supernatants were checked by ring test for excess antibody with GG and for excess of antigen with anti-M serum. The supernatants containing excess antibody were further absorbed with GG until ring tests were negative with GG. These latter supernatants were then tested with a solution of M for the presence of specific antibody.

As a further test for the specific antigenicity of macroglobulins the following experiment was performed (experiment B). To a 3-ml. aliquot of anti-M serum, GG was added in sufficiently high concentration to inhibit any precipitation (45 ml. of 2.5 per cent solution) between GG and anti-M serum. In a series of 12 tubes, 1-ml. aliquots of this solution were incubated with equal volumes of M in halving dilutions, in concentrations varying from 15 mg. per ml. to 8 γ per ml. In a series of control tubes GG was substituted for M. In another experiment the procedure was repeated using, instead of GG, a solution of concentrated serum proteins (20 per cent in saline), isolated from normal human serum by precipitation with ammonium sulphate at 66 per cent saturation.

[4] Batch 88 Squibb Co., obtained through the courtesy of the American National Red Cross, Washington, D. C.

Agar gel techniques

To determine the minimum number of antigenic moieties in each antigen solution, the degree of cross-reactivity of the various systems and the presence of specific antigenic groups or antigens in the macroglobulin fraction, the following agar gel techniques were used.

Oudin tubes were set up for "single diffusion" (19). The antisera were diluted with 3 volumes of a 1 per cent solution made up in saline. All the antigen solutions used in these experiments were made up to 2 per cent in saline. Each antiserum was tested with the 3 antigen solutions: M, NHS, and GG.

The method recently described by Oudin (20) using glass cells with parallel walls was used to determine the antigenic similarities of M, GG and NHS. In these experiments the antisera and antigen solutions were diluted with equal volumes of 2 per cent agar. The concentrations of the GG, M and NHS were 1.8 per cent, 1.5 per cent and 3.5 per cent, respectively. The lower section of the cell was filled with agar solution containing the antiserum, the central section with agar only, and each of the two halves of the upper section with one of the antigens in agar (see Figure 7).

In order to ascertain further whether the macroglobulin fraction contained any specific antigenic moieties not present in normal serum, an experiment similar in principle to experiment B (described above) was devised. A solution of the normal serum proteins was made up to a concentration of 20 per cent in the agar used for the Oudin double diffusion method. This solution, referred to hereafter as AP, was used instead of pure agar in all compartments of the Oudin cell for "double diffusion." Thus a uniform concentration of normal serum proteins was maintained throughout the whole cell. Anti-M serum in AP was placed in the lower compartment and M in AP was placed in the antigen compartment.

In a control cell the upper section was divided into three segments (Figure 8B). Segments I and II were filled with solutions of macroglobulins in AP, the concentrations of M being 0.75 per cent and 0.38 per cent, respectively. Segment III contained only AP solution.

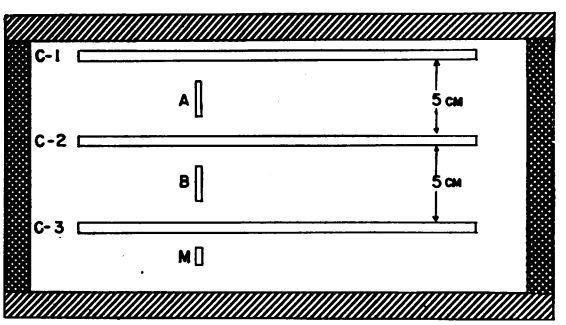

FIG. 1. IMMUNO-ELECTROPHORESIS

A and B represent ditches containing the antigen solution to be separated; C-1, C-2 and C-3 represent channels containing the antisera; M represents the ditch for the "marker" solution.

Ouchterlony plates (19) with four circular wells symmetrically placed with respect to each other in a Petri dish of 9-cm. diameter were used. Two wells, diametrically opposed, were filled with anti-M and anti-GG, and the two other wells with the homologous antigens. The concentrations of the reactants were the same as in the previous experiment.

Immuno-electrophoresis was performed according to the method of Williams and Grabar (21). The glass plates used were 30 cm. × 15 cm. and were covered with transparent films of agar, as described by Oudin (22). Electrophoresis was carried out in an agar medium (1 per cent agar in veronal buffer at pH 8.6 and ionic strength 0.025). The height of the agar layer was 3 mm. In view of the high degree of electro-osmosis in agar, the material to be separated was applied in most experiments, in small ditches (20 mm. × 4 mm. × 3 mm.) 10 cm. from the anode (A and B in Figure 1). Two antigen solutions made up in agar [5] were separated simultaneously on each plate and after electrophoresis the antisera in agar were placed into 5 mm. wide channels (C) which were cut longitudinally parallel to the direction of electrophoretic separation (Figure 1). To follow visually the electrophoretic separation a "marker" (M) was used. This consisted of normal serum mixed with small amounts of bromphenol blue (travelling slightly ahead of the albumin) and hemoglobin (travelling with the β-globulins.) The electrophoresis was carried out in a well-insulated box to minimize evaporation. The electrode vessels were identical to those used for paper electrophoresis. The voltage applied across the plate was 30 to 45 volts and the current was about 20 mA. The duration of an experiment was 24 to 30 hours during which time the albumin and γ-globulin were separated over a distance of 15 cm. The distribution of the separated proteins was established with the help of a "print," taken as described for starch electrophoresis (18). Immediately after filling channels C-1, C-2 and C-3, the agar plates were covered with glass plates and sealed with silicone grease. The plates were then placed on a level surface at room temperature and observed intermittently for the formation of precipitin bands.

RESULTS

Free electrophoresis

The results of free electrophoresis for the four sera and for the respective supernatants (serum minus macroglobulins) are given in Table I together with the results obtained in this laboratory for normal sera. As can be seen the γ-globulin fractions of all four sera were highly elevated. However, only the first three sera contained appreciable quantities of macroglobulins. The mac-

[5] Two volumes of the antigen solution were mixed with one volume of a 3 per cent agar solution in veronal buffer of ionic strength 0.075.

TABLE I

Free electrophoresis

Case		Relative percentages					Gm. per 100 ml. in whole serum	
			Globulins					
		Albumin	Alpha-1	Alpha-2	Beta	Gamma	Total protein‡	Macro-globulins§
C. V. (Female)	W*	33.1	8.8	19.8	11.8	26.5	7.38	2
N. R. (Male)	W	16.8	4.8	7.2	12.7	58.5	10.68	5
	S	41.4	10.2	5.9	9.6	32.9		
A. B. (Female)	W	26.8	3.6	7.1	8.9	53.6	16.10	8
	S	61.6	5.0	12.7	12.6	7.1		
L. S. (Female)	W	31.3	3.2	7.0	9.2	49.3	12.18	0.7
	S	34.0	3.4	7.0	9.2	46.4		
Normal† human serum	Male	59.4 ± 3.2	4.7 ± 1.8	8.6 ± 1.4	12.9 ± 1.3	14.3 ± 2.5	8.12 ± 0.59	
	Female	58.4 ± 2.3	5.2 ± 0.7	9.8 ± 1.0	12.4 ± 1.2	14.2 ± 1.8	7.92 ± 0.71	

* W—Whole unfractionated serum, S—Supernatant (serum minus macroglobulins).
† Average values as found for 22 male and 14 female sera ± one standard deviation.
‡ Determined refractometrically.
§ Approximate values.

roglobulin fraction of each of three sera (C. V., A. B. and L. S.) gave rise to single symmetrical peaks with mobility values of 0.64, 1.10 and 0.79 cm.^{2} per volt per sec., all of which fell below the range of those for normal γ-globulins (1.23 to 1.67 cm.^{2} per volt per sec.) The macroglobulin fraction of the fourth serum (N.R.) resolved itself into three peaks with mobilities (1.12, 3.29, 4.70 cm.^{2} per volt per sec.) corresponding to those of γ-, β- and α_2-globulins. The macroglobulin fraction of the serum A.B. could not be resolved by free electrophoresis into more than one peak within the pH range 3.6 to 8.6. Figure 2 represents the patterns obtained by free electrophoresis for the whole sera A.B. and N.R. and their macroglobulins and supernatants.

Paper electrophoresis

The results of paper electrophoresis confirmed those obtained by free electrophoresis. Staining of the electrophoretograms of the whole sera with fuchsin revealed, in addition to the fuchsin stainable bands found in normal sera, the presence of material rich in carbohydrate in the γ-globulin region. The fuchsin stainable band in the γ-globulin region appeared to be associated with the macroglobulins, as evidenced by its presence on the electrophoretograms of the macroglobulin solutions and its absence in the supernatants.

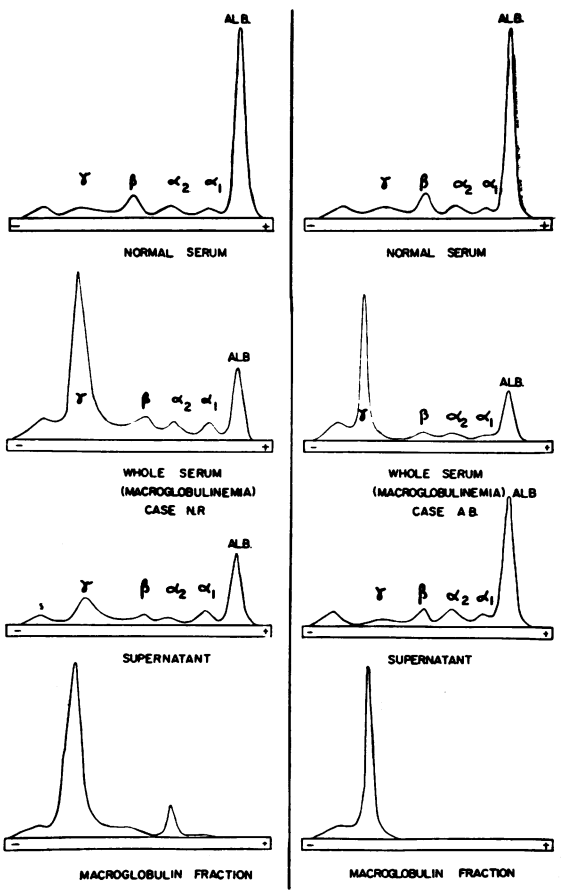

FIG. 2. SEPARATION BY FREE ELECTROPHORESIS

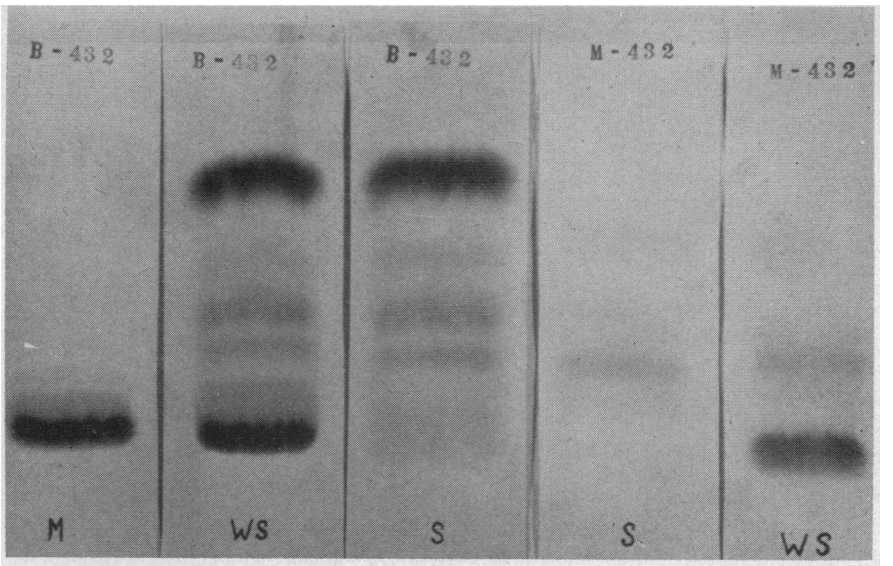

FIG. 3. PAPER ELECTROPHORESIS OF SERUM A.B.

WS represents whole serum; M represents macroglobulin fraction; S represents supernatant. The three strips on the left are stained with Amido-black 10B for proteins, while the two on the right are stained with periodic acid Schiff reagent for carbohydrates.

Figures 3 and 4 represent the electrophoretograms of the whole sera of A.B. and N.R. and their corresponding macroglobulins and supernatants stained for protein and carbohydrate.

Separation of A.B. serum by starch electrophoresis

The resolution of this serum by starch electrophoresis is illustrated in Figure 5. Each of the eluted fractions was examined by both paper electrophoresis and ultracentrifugation. Four of the eluted fractions, albumin, α_1-, α_2- and β-globulins, displayed normal migratory and staining properties by paper electrophoresis. Ultracentrifugation revealed a rapidly sedimenting material (16.4 S) in low concentration associated with the α_2 fraction. The four γ-globulin sub-fractions were all heterogeneous in the ultracentrifuge. Three of the latter fractions (B, C, D,) were composed of normal and macroglobulin components. Only one fraction (A) appeared to be free of slowly sedi-

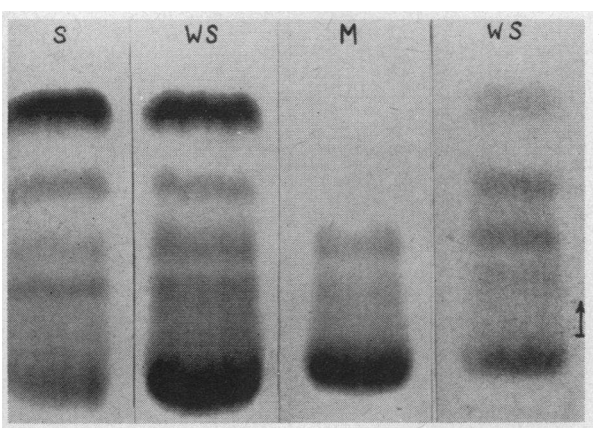

FIG. 4. PAPER ELECTROPHORESIS OF SERUM N.R.

WS represents whole serum; M represents macroglobulin fraction; S represents supernatant. The three strips on the left are stained with Amido-black 10B for proteins, the fourth strip is stained with periodic acid Schiff reagent for carbohydrates.

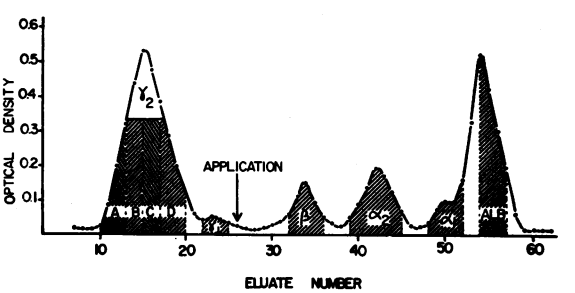

FIG. 5. SEPARATION OF SERUM A.B. BY STARCH ELECTROPHORESIS

The shaded areas represent fractions eluted from the starch block. A, B, C and D represent subfractions of γ_2-globulins.

TABLE II

Sedimentation constants of serum fractions obtained by starch electrophoresis (serum A.B.)

Gamma-2								
A	B	C	D	Gamma-1	Beta	Alpha-2	Alpha-1	Albumin
	7.3	6.5	6.5	7.5	4.8	6.7	3.7	4.0
16.4	15.2	15.7	14.6			16.5		
23.2	21.7	22.0	22.2					
	28.8	28.1	28.8					

menting γ-globulin (Table II). The data of Table II would suggest that the macroglobulins migrate at a somewhat slower rate in an electric field at pH 8.6 than do the γ-globulin constituents regularly found in normal serum.

Ultracentrifugation

The macroglobulin fractions of the four sera possessed components of high sedimentation constants. The results of ultracentrifugal analyses are presented in Table III. The macroglobulin fraction of serum N.R. resolved itself into a broad spectrum of components with sedimentation constants of 3.4, 6.0, 11.0, 12.6, 17.7 and 27.5 S. By far the greater part of the macroglobulin fraction (82 per cent) was composed of the fast sedimenting material (peaks with S values of 11 to 27.5). The macroglobulins in serum A.B. (Figure 6) also appeared to be heterogeneous ultracentrifugally, possessing sedimentation constants of 16.4, 23.3, 28.8 S, in addition to a small peak with sedimentation constant of 7.4 S. These values correspond to the S values determined for the four γ-globulin subfractions isolated by starch electrophoresis. The macroglobulins of serum L.S. had sedimentation constants of 6.5, 10.6 and 15.3 S. Sedimentation constants of 6.5, 18.5 and 26.0 were calculated for the components in the macroglobulin fraction of serum C.V.

TABLE III

Ultracentrifugal analysis of the macroglobulins

Case	Sedimentation constants*	Percentages†
C. V.	6.5	55.0
	18.5, 26.0	45.0
N. R.	3.4	5.8
	6.0	11.9
	11.0	11.3
	12.6, 17.7	61.2
	27.5	9.8
A. B.	7.4	4.6
	16.4	56.5
	23.3	32.2
	28.8	6.7
L.S.	6.5	57.5
	10.6	22.4
	15.3	20.1

* In Svedberg units.
† Relative distribution of the components in the macroglobulin fractions.

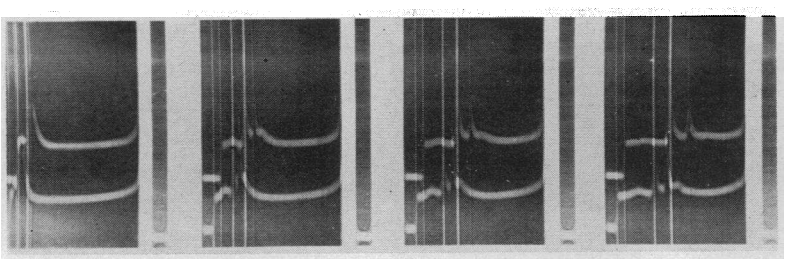

FIG. 6. ULTRACENTRIFUGATION OF SERUM A.B.

The lower pattern represents macroglobulins in 1:2 dilution analyzed in a standard cell, the upper pattern represents macroglobulins in 1:3 dilution in a cell provided with a wedge disc. The above frames (from left to right) were photographed at 10, 17, 22 and 28 min. after the rotor attained full speed of 59,780 r.p.m.

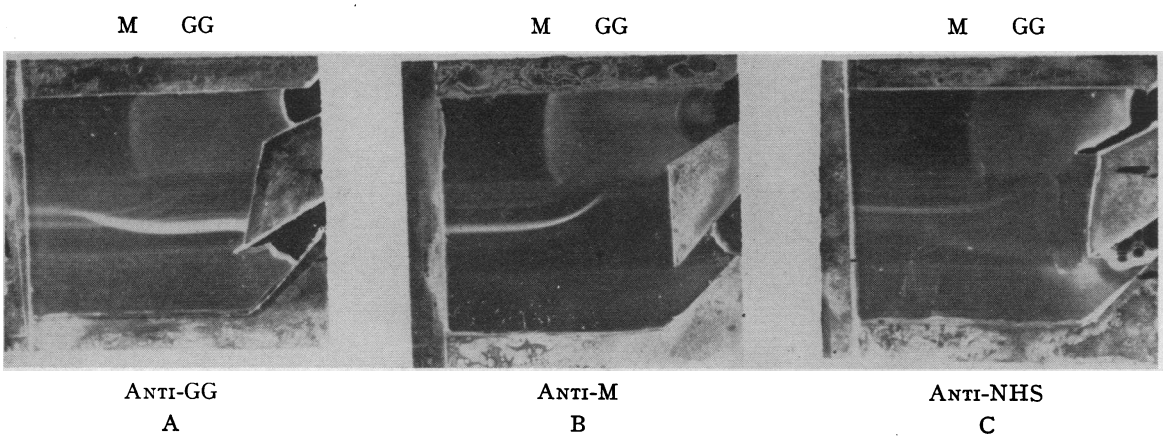

FIG. 7. DOUBLE DIFFUSION EXPERIMENTS IN OUDIN CELLS WITH PARALLEL WALLS

M represents macroglobulin fraction of serum A.B., GG represents γ-globulin; Anti-M represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum; Anti-NHS represents rabbit anti-normal human serum.

Immunologic results

1. *Precipitin method*—In experiment A precipitation occurred in tubes 2 to 18 in the series of 19 tubes. Supernatants from tubes 2 to 18 gave positive ring tests with GG and those from tubes 1 to 12 gave positive ring tests with anti-M serum. The supernatants were subsequently absorbed with GG until antibody could no longer be detected against GG. However, the supernatants still gave positive ring tests with M, thus demonstrating the presence of a precipitating antibody-antigen system specific to M.

In experiment B, precipitates were formed only in tubes 5 to 10 of the series of 12 tubes. The optimum zone appeared to be in tube 8. Similar results were obtained when the proteins of normal serum were substituted for GG, the optimal zone being observed in tube 9.

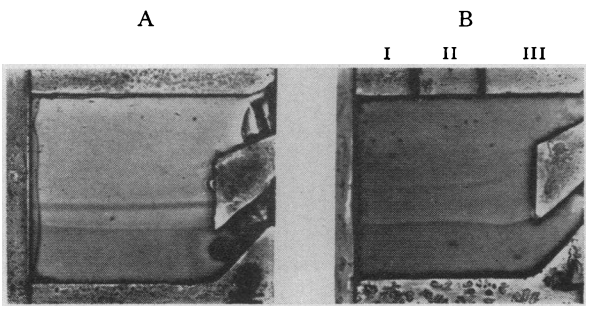

FIG. 8. DOUBLE DIFFUSION EXPERIMENTS IN OUDIN CELLS WITH PARALLEL WALLS

The cells represent the system of macroglobulins with rabbit anti-macroglobulin serum in presence of an excess of normal serum proteins.

Agar gel techniques

Precipitin bands were formed in all Oudin tubes indicating thus that M, GG and NHS contained some common antigenic moieties.

The results of the experiments using the Oudin cells with parallel walls (Figure 7) demonstrate the extent of antigenic similarity of M and GG.[6] In Figure 7A it appears that both M and GG contained at least three identical antigenic moieties. In plate 7B seven bands were formed between M and anti-M, two of which were common to the GG-anti-M system. As revealed in Figure 7C a minimum of four bands was formed only between M and anti-NHS and three additional bands were common to the two systems. Figure 8 illustrates the results for the M-anti-M system in agar in the presence of an excess of normal serum proteins. As can be seen in Figure 8A one distinct band was produced across the central portion of the cell when the upper section was filled completely with macroglobulin in AP solution. This result indicates the presence of at least one specific antigenic moiety in the macroglobulin fraction which is absent from normal serum. This conclusion is further supported by the discontinuity of the band

[6] The bands were much more clearly delineated in the agar than in the photographic reproductions.

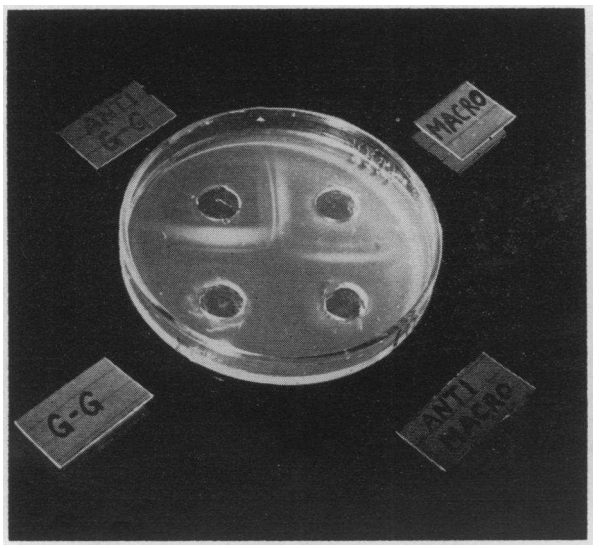

FIG. 9. OUCHTERLONY PLATE

Macro represents macroglobulin fraction of serum A.B., GG represents γ-globulin; Anti-macro represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum.

in the region containing only the AP solution in the control experiment (Figure 8B).

The Ouchterlony plate is shown in Figure 9. M versus anti-GG gave rise to four bands while each of the homologous antigen-antibody systems gave rise to two bands only. GG gave only a faint band against anti-M.

The immuno-electrophoretic results are illustrated in Figures 10 and 11.

Electrophoresis of the NHS in agar followed by the application of the anti-M, anti-NHS and anti-GG sera in the longitudinal channels resulted in the formation of a large number of precipitin bands (Figure 10). The anti-GG reacted with the electrophoretically separated NHS proteins to yield a long continuous precipitin band extending from the γ-globulin region into the albumin region, in addition to two faint bands in the β-globulin and albumin regions. The long precipitin band was also formed by the NHS anti-NHS system but not by the NHS anti-M system. In all other respects, the anti-M and anti-NHS appeared to form the same number of bands similar in their distribution. The absence of bands between anti-M and NHS (in Figure 10) and between anti-M and GG (in Figure 11) in the region of γ-globulins is somewhat perplexing in view of the copious precipitates obtained when anti-M was incubated with GG as previously mentioned. When M was separated by electrophoresis in the agar and anti-M

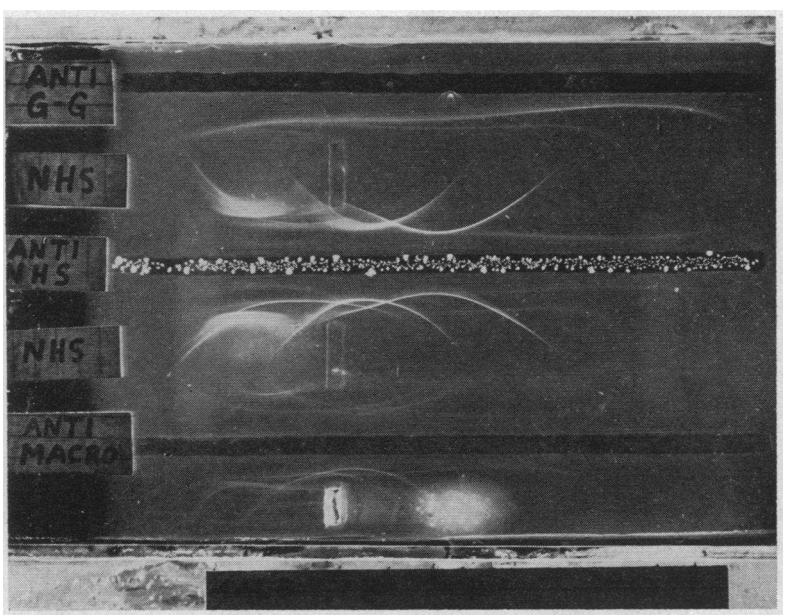

FIG. 10. IMMUNO-ELECTROPHORESIS

NHS represents pooled normal human serum; Anti-GG represents rabbit anti-γ-globulin serum; Anti-NHS represents rabbit anti-normal human serum; Anti-Macro represents rabbit anti-macroglobulin serum.

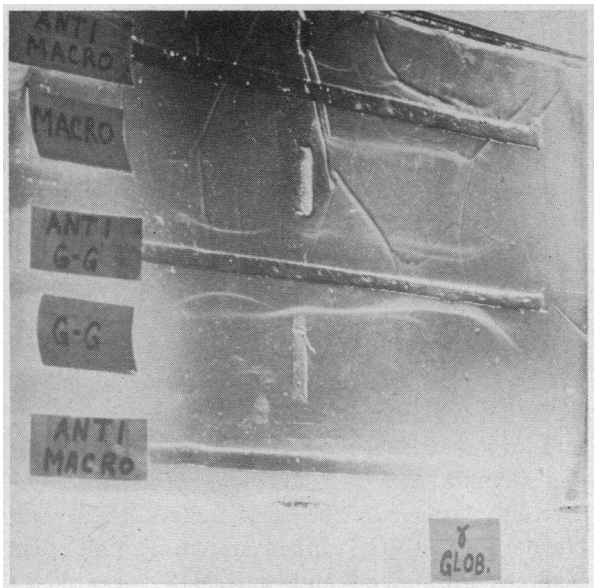

FIG. 11. IMMUNO-ELECTROPHORESIS

Macro represents macroglobulin fraction of serum A.B.; GG represents γ-globulin; Anti-Macro represents rabbit anti-macroglobulin serum; Anti-GG represents rabbit anti-γ-globulin serum.

and anti-GG were deposited into the lateral channels, bands resulted in the γ-globulin region with the anti-M serum and in the albumin, α- and γ-globulin regions with the anti-GG serum. On the other hand, a long continuous heavy band was formed between the electrophoretically separated GG and anti-GG which extended from the γ-globulin region into the albumin region. In addition, a small, faint but distinct band was detected in the albumin region.

One of the difficulties encountered during the immuno-electrophoretic experiments was the low solubility of the macroglobulins in the buffer used for the immuno-electrophoresis. The macroglobulins (A.B.) were essentially insoluble in veronal buffer of ionic strength 0.025 and pH 8.6. However, the use of veronal buffer of higher ionic strength, in which the macroglobulins were soluble, resulted in the passage of higher current, accompanied by increased evaporation of water from the agar. This effect resulted in deformation of the agar gel. Therefore, veronal buffer of ionic strength 0.025 was resorted to even though some elements of the macroglobulin solution precipitated about the zone of application.

DISCUSSION

Although the macroglobulins of three sera gave rise to only single symmetrical peaks in the Tiselius cell and appeared to be slow moving γ-globulins, ultracentrifugal analysis demonstrated the high degree of heterogeneity of these fractions. The macroglobulins of the A.B. serum could not be resolved by free electrophoresis at different pH's into more than one component. However, by ultracentrifugation this fraction was shown to contain at least four easily distinguishable components (S values of 7.4, 16.4, 23.3 and 28.8 S). The macroglobulins of only one serum (N.R.) were resolved by free electrophoresis into three components. This to our knowledge is the first case displaying electrophoretically heterogeneous macroglobulins.

The results of paper electrophoresis showed clearly the presence of carbohydrate rich material in the macroglobulin fraction as revealed by the Schiff reagent. In general, only a faintly visible carbohydrate band is detectable in the γ-globulin region of normal sera.

Recently, Wallenius, Trautman, Franklin, and Kunkel (23) reported that the "19 S" heavy components normally found in human serum were associated with the α_2-globulins (65 per cent) and with β- and γ-globulins (35 per cent). In addition they found small amounts of heavier components (28 S and 44 S) to be distributed throughout the γ-globulin region. The results of our ultracentrifugal analyses of the fractions of serum A.B., isolated by starch electrophoresis, confirm in part the results of the above workers. As illustrated in Table II the α_2-globulins of serum A.B. which were not water precipitable contained a heavy component with a sedimentation constant of about 16.5 S. Heavy components were also found throughout the entire γ_2-globulin region as in the experiments of other workers (23) but were absent in the γ_1-globulin region. Moreover, the electrophoretically slowest components of γ_2-globulins appeared to contain only fast sedimenting materials (fraction A of Table II). Thus, on the basis of our physico-chemical results, it is not possible to arrive at a clear-cut conclusion whether macroglobulins are distinct serum moieties synthesized during the course of the disease (Waldenström syndrome) or whether they are the result

of a greatly accelerated synthesis of a moiety present in normal human serum in only negligible amounts.

On the other hand, the immunologic results tend to indicate that macroglobulins contain specific antigens (or at least specific antigenic groups) not present in normal serum. The fact that anti-M serum gave positive ring tests with NHS, GG and M demonstrates that the material precipitated with distilled water from the sera containing macroglobulins possessed at least some antigens identical to those found in normal human serum. As no single zone of optimal proportions was detected in the precipitin test between anti-M serum and GG, it can be assumed that both M and GG are multiantigenic systems. The findings that precipitation occurred over a wide range of antigen concentrations (from 45 mg. per ml. to 0.6 γ per ml.) support this interpretation.

In an attempt to demonstrate the presence of antigenically specific moieties in M, experiment B was performed. As is generally known, specific precipitation in a solution containing a number of antigen and antibody systems can be prevented by having all the antigens in great excess. In experiment B, anti-M serum was mixed with a sufficiently high excess of GG to inhibit precipitation. Titration of this solution with M resulted in precipitation occurring only in the six central tubes of the precipitin series of 12 tubes. This precipitation can be attributed to the presence of an antigenic factor or factors present in the macroglobulins and absent in the γ-globulin preparation used.

However, the possibility still existed that this factor might have been associated with any of the other normal serum protein fractions. A concentrated solution of normal serum proteins was substituted for the γ-globulins in the above experiment. As previously stated, no precipitation occurred between the anti-M serum when incubated with a concentrated solution of normal serum proteins. Titration of this solution with M resulted in precipitation occurring only in six tubes, the Dean and Webb optimal zone being only one tube removed from that obtained with GG. Therefore, the precipitation which occurred on addition of the macroglobulin solution to the anti-M serum in presence of an excess of GG or normal serum proteins must be attributed to some antigenically specific entity associated with the macroglobulins.

The Oudin technique, using the cell with parallel walls, allows one to demonstrate the presence of identical antigens and/or antibodies in different solutions. The results obtained with this technique (multiplicity of bands in the systems illustrated in Figure 7) confirm the conclusion arrived at on the basis of the classical precipitin method that both M and GG are complex systems containing several immunologically identical antigens and possibly some specific antigens as well. Since a precipitin band was obtained with the M-anti-M system in the presence of an excess of normal serum proteins (Figure 8), one could conclude that our macroglobulin fraction contained some antigenically specific material absent from normal serum. In essence, these results corroborate the results of Experiment B.

Immuno-electrophoresis was used in the hope that it would reveal whether the different antigens present in M solution were associated with distinct serum proteins separable by electrophoresis. The results of Figure 10 (NHS-anti-M system) would suggest that M possessed most of the antigens present in normal serum. On the other hand the formation of the bands in the γ-globulin region of Figure 11 between M and anti-M confirms the results of the ultracentrifugal analyses of the fractions obtained by starch electrophoresis, demonstrating that the water precipitable heavy component was principally associated with γ-globulins. The formation of additional bands in the region of the albumin and α-globulins in Figure 11 between M and anti-GG on the one hand and GG and anti-GG on the other would suggest that both M and GG contained trace amounts of some antigenic constituents having mobilities of albumin and α-globulins in addition to moieties with properties similar to those of γ-globulins. These results are noteworthy as neither paper electrophoresis nor free electrophoresis of the M or GG revealed the presence of any albumin or α-globulin components, and would thus confirm the claims of Oudin and Grabar (20, 21) that the use of gel technique allows for the detection of minute amounts of antigen or antibody.

The immuno-electrophoresis of NHS in agar yields with anti-NHS and anti-GG a number of

short bands in addition to a long continuous band extending from the γ-globulin region into the albumin region (Figure 10). This band is most pronounced in the γ-globulin region. This result can be explained in two ways. It may be due to cross-reactivity between GG and all the other proteins normally found in serum. This would imply that the other serum proteins contain some antigenic groups identical to those of γ-globulins. The other explanation would be that γ-globulins are preferentially absorbed onto the agar matrix during their migration by electro-osmosis from the zone of application to their final location on the agar plate.

On the basis of the immuno-electrophoretic results obtained with the GG-anti-GG system (Figure 11) it would appear that the first hypothesis might be ruled out. For, as can be seen in Figure 11 a long continuous band similar to the bands obtained with the NHS-anti-NHS or anti-GG system (Figure 10) was also formed between the γ-globulins and anti-GG. This band also extended from the γ-globulin region into the albumin region. However, in view of the contradictory results obtained recently by Slater (24) who claims to have detected some antibody titer associated with β- and α-globulin in addition to the bulk of the antibody residing in γ-globulins, both factors, *viz.*, cross-reactivity of the different serum proteins and absorption, might be partly responsible for the formation of the precipitin band throughout almost all of the protein spectrum. A further point that deserves mention is the reliability of the agar gel techniques in detecting combination between antigen and antibody. Since the anti-M and anti-GG sera formed several clearly delineated bands with either of the two antigens (M or GG) in the Oudin cells (Figure 7), and since copious precipitates were obtained on incubating anti-M with GG in saline, the appearance of only faint lines by immuno-electrophoresis for the anti-M and GG system (Figure 11) is somewhat perplexing. Similarly, only faint bands were formed between anti-M and GG in the single diffusion Oudin tubes and on the Ouchterlony plate (Figure 9). It should be emphasized that although agar gel techniques will, in general, detect trace amounts of antigen or antibody in some systems, in other cases—for reasons which we do not know as yet—almost complete inhibition of precipitin bands may occur.

SUMMARY

Sera from four cases of macroglobulinemia (Waldenström syndrome) were investigated by means of zone (paper and starch) and free electrophoresis, ultracentrifugation and immunologic methods (precipitin technique and the Oudin, Ouchterlony, and Grabar agar gel technique).

On free electrophoresis the macroglobulin fraction of three sera gave rise to single symmetrical peaks with mobility of a slow moving γ-globulin, while the macroglobulin fraction of the fourth serum was resolved into three peaks with mobilities of α_2-, β- and γ-globulins.

The paper electrophoretograms revealed the presence of material rich in carbohydrate associated with the macroglobulin fractions.

The ultracentrifugal analyses demonstrated that the macroglobulin fractions were heterogeneous, the sedimentation constants of the different constituents varying from 10.6 to 28.8 S.

The immunologic methods indicated the presence of antigenically specific material in the macroglobulin fraction which is absent from normal serum.

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