*Immunology*, 1970, **19**, 909.

# Precipitin Reactions of the C1q Component of Complement with Aggregated γ-Globulin and Immune Complexes in Gel Diffusion

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(*Received* 3rd *March* 1970)

**Summary.** A gel diffusion method for demonstrating precipitin reactions of C1q with aggregated γ-globulin and immune complexes is described. Optimal precipitin lines were found to occur with 0·6 per cent agarose in 0·01 M EDTA at pH 7·2 and ionic strength 0·1. Reduction and alkylation of γ-globulin aggregates destroyed the precipitability with C1q. Precipitation occurred only with aggregates greater than 19S and with soluble immune complexes formed in two to twenty times antigen excess. γ-Globulin complexes were detected by this procedure in hypocomplementaemic sera from patients with systemic lupus erythematosus and hypocomplementaemic joint fluids from patients with rheumatoid arthritis. The relevance of this *in vitro* system to *in vivo* complement consumption in various disease states is discussed.

## INTRODUCTION

The C1q component of complement contains the site through which the first component combines with γ-globulin or specific antibody (Müller-Eberhard and Calcott, 1966; Müller-Eberhard, 1968). Under proper conditions C1q in normal serum can be precipitated by soluble γ-globulin aggregates (Müller-Eberhard and Kunkel, 1961). The direct reaction with γ-globulin can also be demonstrated by analytical ultracentrifugation (Müller-Eberhard and Kunkel, 1961), agglutination of γ-globulin coated latex particles (Ewald and Schubart, 1966), and most recently by a gel diffusion method (Agnello, Carr, Koffler and Kunkel, 1969). The present paper describes in detail the latter method and its application in studying the nature of C1q interactions with aggregated γ-globulin. An additional application described is the use of C1q in gel diffusion to detect γ-globulin complexes in pathological body fluids.

## MATERIALS AND METHODS

### *Gel preparation*

Agarose gel was prepared with Seakem agarose (Marine Colloid, Inc.) in buffer containing sodium azide 0·001 per cent. The solution was filtered through No. 2 Whatman paper and 22 ml were pipetted into a 9 cm plate to give a 5 mm thick gel. Wells were cut 9 mm in diameter with a centre to centre distance between wells of 12 mm. Agar-agar (Baltimore Biological Laboratory, Inc., Baltimore, Maryland) gel was prepared in a similar way.

Gels at 0·05 ionic strength were prepared using the following buffer systems: pH 9·4, pH 8·6, pH 7·8 diethylbarbituric acid, sodium diethyl barbiturate; pH 7·2, pH 6·0 cacodylic acid, sodium cacodylate; pH 5·0, pH 4·0 acetic acid, sodium acetate.

Buffers were adjusted to higher ionic strengths by addition of solid sodium chloride. Tetra sodium EDTA, one molar solution, was used to make gel buffers containing EDTA.

*Preparation of C1q*

C1q was isolated from normal serum or recalcified plasma by precipitation with calf thymus deoxyribonucleic acid (DNA) (Worthington Biochemical Corporation) (Agnello, *et al.*, 1969). Pooled serum was dialysed overnight against pH 8·6, 0·025 M veronal, 0·01 M EDTA buffer. A precipitin curve was made of the reaction of the serum and DNA to determine maximal precipitation. The optimal amount of DNA, usually 25 μg per ml, was then added to the pooled serum, stirred at room temperature for 1 hour and kept at 4° for 24 hours. The solution was spun at 1000 ***g*** for 30 minutes and the precipitate obtained washed four times with pH 8·6, 0·025 M sodium veronal buffer and resuspended in pH 6·9, 0·05 M phosphate, 0·003 M magnesium chloride, 0·05 M saline buffer. The pH was adjusted to 6·9 with 1 N hydrochloric acid. 100 μg DNAase 1 (Worthington Biochemical Corp.) was added per ml of suspension. The suspension was then stirred at room temperature for 3 hours and dialysed against the suspending buffer until most of the precipitate dissolved. The solution was spun at 100,000 ***g*** for 30 minutes. The supernatant contained approximately 70 per cent C1q and was further purified by column chromatography using G-200 Sephadex in pH 5·3, 0·3 M phosphate buffer (Müller-Eberhard, 1968) which also removed the DNAase and DNAase digestion products. The column purified material contained greater than 90 per cent C1q by radial immunodiffusion and analytical ultracentrifugation. The latter preparation was used at 0·2 mg/ml in diffusion plates.

*Preparation of aggregated FII γ-globulin and immune complexes*

Aggregated γ-globulin was prepared from FII human γ-globulin (Lederle Laboratories) as previously described (Müller-Eberhard and Kunkel, 1961). By analytical ultracentrifugation, the final preparation contained no detectable 7S γ-globulin. Aggregated γ-globulin was used at 1 mg/ml in diffusion plates.

Reduced and alkylated aggregated γ-globulin was prepared by incubating aggregated γ-globulin at 10 mg/ml for 1 hour in 0·2 M 2-mercaptoethanol and alkylated with a 10 per cent excess of iodoacetamide. The preparation was then dialysed against pH 8·6, 0·065 M veronal buffer.

Aggregated γ-globulin was fractionated by sucrose density gradient ultracentrifugation as reported earlier (Kunkel, 1960). A linear 10–40 per cent sucrose gradient in pH 7·2 phosphate buffered saline was used. 0·2 ml of heat-aggregated γ-globulin at 200 mg/ml was applied to the gradient and spun at 50,000 ***g*** for 17 hours at 4°. An isolated γM myeloma protein and *E. coli* alkaline phosphatase (Worthington Biochemical Corp.) were used as the 19S and 6·5S markers respectively. The γM marker was immunologically assayed and the alkaline phosphatase activity was determined as previously described (Olins and Edelman, 1964).

The globulin fractions of pooled rabbit antisera to human serum albumin (HSA) were prepared by precipitation at 40 per cent saturation with neutralized ammonium sulphate and purified further by DEAE column chromatography. The antibody content

of this final preparation was 1·8 mg of antibody protein per ml. Precipitin curves were prepared with 0·2 ml of antibody solution and increasing amounts of HSA from 10 μg to 5 mg. The precipitates obtained were washed twice with 0·9 per cent saline at 4°, dissolved in 0·1 ml of 0·1 N sodium hydroxide, and the protein quantified by the Folin-Ciocalteu method. The supernatants were tested for precipitation with C1q by gel diffusion.

Isolated 19S rheumatoid factor prepared by the method of Schrohenloher, Kunkel and Tomasi (1964) was used at a concentration of 1 mg/ml.

Antisera specific for C1q were raised in rabbits by injection of the purified material described above in incomplete Freund's adjuvant. In addition, an anti-serum kindly supplied by Dr H. J. Müller-Eberhard was used.

*Joint fluids and sera*

Joint fluids from patients with rheumatoid arthritis and other diseases were collected under sterile conditions, allowed to clot, treated with hyaluronidase, centrifuged to remove debris, and stored at 4°. Cryoprecipitates which were formed over several days were removed by centrifugation in most instances.

Sera obtained from normal donors and patients with various diseases were used fresh or after freezing and storage at −70°.

Sucrose density gradient ultracentrifugation of serum was carried out as for the separation of aggregated γ-globulin except that the gradient was spun at 100,000 ***g*** for 18 hours.

Precipitin curves of the reaction of 20 μg C1q with increasing amounts of pathological sera were carried out at pH 8·6 in the presence of 0·01 M EDTA at 0·09 ionic strength. The protein content of the washed precipitate was determined as above.

## RESULTS

### OPTIMAL CONDITIONS FOR REACTION OF C1q WITH AGGREGATED GLOBULIN

The reaction of normal serum and isolated C1q with aggregated globulin and anti C1q rabbit serum under optimal conditions is shown in Fig. 1. The common line of precipitation produced by these reactions indicates that it is the C1q in normal serum that reacts with aggregates. Heating normal serum or the isolated C1q to 56° for 20 minutes eliminates the precipitin reaction with aggregates.

The conditions required for these reactions were studied in detail. The effect of gel type, pH, ionic strength, and EDTA were tested. Precipitin lines were obtained at pH

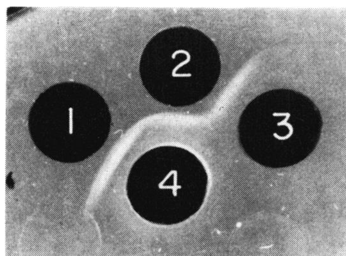


Fig. 1. Precipitin lines in agarose between aggregated γ-globulin and isolated C1q or C1q in normal serum. 1, Anti-C1q; 2, aggregated γ-globulin; 3, purified C1q; 4, normal human serum.

9·4–6·0, although at the lower pH lines developed more slowly. pH 7·2 was optimal for most purposes because smaller quantities of aggregated γ-globulin could be detected and precipitin lines were, in general, sharper. Good precipitin lines were obtained in gels at 0·05–0·125 ionic strength. A weak reaction could be obtained at 0·20 *I* but no precipitation occurred at higher ionic strength. A faint line of precipitation occurred between Clq and normal serum at ionic strengths below 0·08. Sera were usually diluted two-fold when screening for complexes. The optimal conditions found are listed in Table 1.

Table 1
Optimal conditions for the reaction of Clq with aggregated γ-globulin in gel diffusion

| | |
|---|---|
| Gel | 0·6 per cent Agarose |
| pH | 7·2 |
| Ionic strength | 0·09 |
| Temperature | 22°—48 hours, then 0°—72 hours |
| EDTA | 0·01 molar |

In earlier studies on precipitation of Clq in normal serum with aggregated γ-globulin, EDTA was found to enhance precipitation (Müller-Eberhard and Kunkel, 1961). A similar effect was observed in gel diffusion in the presence of 0·01 M EDTA. This phenomenon was not due to ionic strength differences but appeared to be related to chelation effect of EDTA; addition of 0·01 M calcium choride to the gel eliminated precipitation enhancement. EDTA also enhanced precipitation of isolated Clq with aggregates. However, this effect was not eliminated by addition of calcium.

Optimal fixation of the first component of complement to γG antibody molecules in immune haemolysis is known to occur at 0° (Lepow, Ratnoff and Levy, 1951). A temperature effect in the gel system was tested by incubating gel plates at 0°, 22° and 37° and qualitatively comparing precipitin lines formed. Precipitin lines with aggregates could be obtained at all three temperatures. After 48 hours incubation optimal lines were present in plates incubated at 22°. The poorer lines in plates incubated at 0° appeared to be due to slower diffusion at that temperature since after prolonged incubation the heaviest lines were obtained at 0°. Also, after lines were obtained at 22° and 37° additional intensification could be obtained by further incubation at 0°. Based on these findings, the routine incubation of 48 hours at 22° and 72 hours at 0° was adopted.

The inability of Clq to diffuse in agar-agar in contrast to its diffusibility in agarose has been noted previously (Müller-Eberhard, personal communication). Table 2 compares

Table 2
Precipitation of aggregated FII with Clq

| Ionic strength | Agar-agar* | Agarose* |
|---|---|---|
| 0·050 | 0 | ++++ |
| 0·075 | 0 | ++++ |
| 0·100 | 0 | ++++ |
| 0·125 | + | ++++ |
| 0·150 | ++ | ++ |
| 0·175 | +++ | + |
| 0·200 | + | + |
| 0·250 | 0 | 0 |

*0·6%, pH 7·2.
Lines of precipitation were scored from + to ++++.

the precipitin reaction of isolated C1q and aggregated γ-globulin at various ionic strengths in 0·6 per cent agar-agar and 0·6 per cent agarose gels at pH 7·2. While good precipitin lines are formed in agarose beginning at 0·05 ionic strength, no lines appear in agar-agar until the ionic strength is greater than 0·1. Optimal lines in agarose are present over a broad range of ionic strength, whereas optimal lines in agar-agar are more restricted. In both gels all lines are eliminated at ionic strength greater than 0·2.

### REACTIONS OF C1q WITH AGGREGATED γ-GLOBULIN AND IMMUNE COMPLEXES

It is known that reduction and alkylation of complement fixing 7S antibody and aggregated γ-globulin destroys the ability to fix complement and presumably to react with C1q (Wiedermann, Miescher and Franklin, 1963). Fig. 2 shows the reaction of isolated C1q with aggregated γ-globulin before and after reduction and alkylation. Isolated rheumatoid factor which is also shown reacts with both types of aggregates as expected while C1q reacts only with the untreated aggregates.

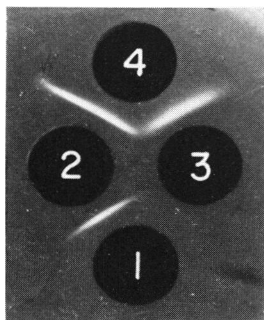


Fig. 2. Effect of reduction and alkylation on the reaction of aggregated γ-globulin with C1q. The reactions with 19S rheumatoid factor are shown for comparison. 1, Purified C1q; 2, aggregated γ-globulin; 3, aggregated γ-globulin reduced and alkylated; 4, purified rheumatoid factor.

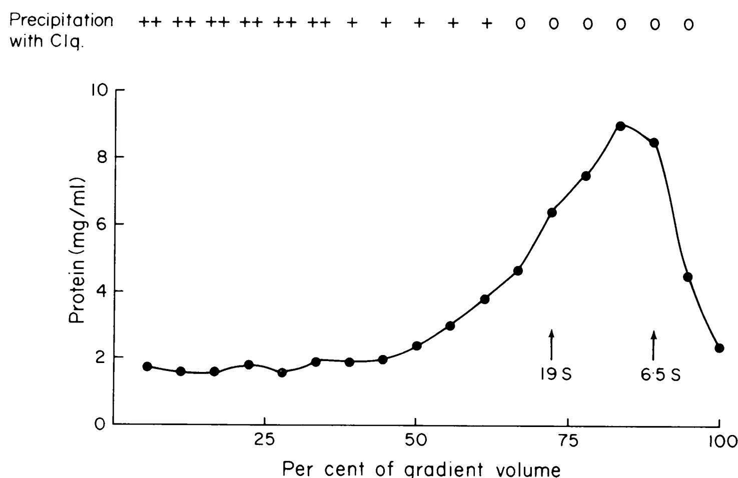


Fig. 3. Sucrose density gradient fractionation of aggregated γ-globulin. Precipitation of various size aggregates with C1q in gel diffusion is indicated at top of figure. The positions of the 19S and 6·5S markers are indicated.

Aggregates of the γG subgroups have been found to differ in their ability to fix complement (Ishizaka, Ishizaka, Salmon & Fudenberg, 1967). Aggregates of γG1, γG2 and γG3 fix complement while γG4 aggregates do not. In gel diffusion aggregates of γG1, γG2 and γG3 proteins gave precipitin reactions with C1q as expected, however, some aggregated γG4 proteins were also found to precipitate with C1q. This could not be explained on the basis of contamination and this question is currently under investigation.

Several workers have presented evidence that larger aggregates of γ-globulin fix complement better than smaller aggregates. Fig. 3 shows a sucrose density gradient of aggregated FII γ globulin. The 6·5S and 19S markers are indicated by arrows. C1q reacts only with aggregates larger than 19S. The strongest reactions occurred with the larger aggregates in the bottom 35 per cent of the gradient volume. A control gradient of reduced and alkylated aggregates produced no precipitation with C1q in any part of the gradient.

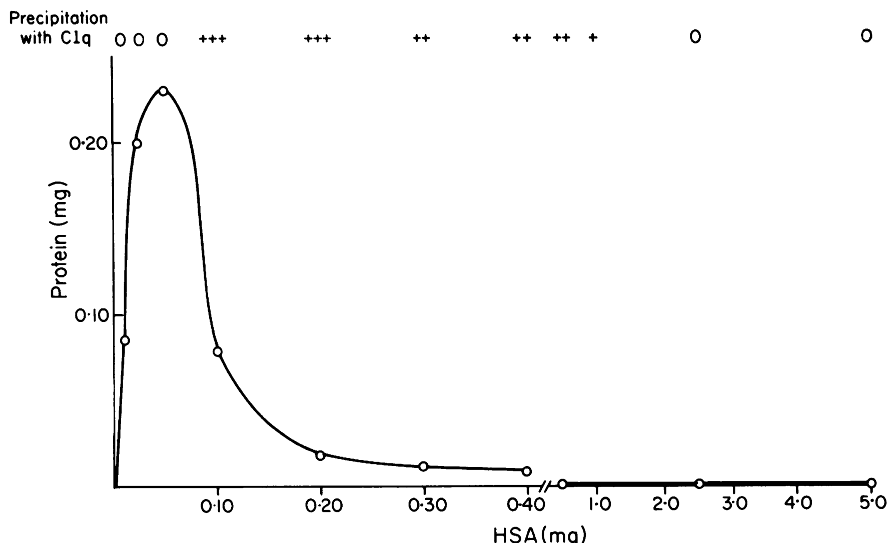


Fig. 4. Precipitin curve of rabbit anti-HSA with HSA. Precipitation of supernatants with C1q in gel diffusion is shown at top of figure.

Precipitation of C1q with immune γ-globulin complexes could also be demonstrated in gel diffusion. Previously, precipitation of *in vitro* formed immune complexes had been demonstrated in solution using total guinea-pig complement (Weigle and Maurer, 1957). The precipitin curve of anti-HSA and HSA is shown in Fig. 4. Precipitation of C1q with the supernatants at various points along the curve is shown at top of the figure. No precipitin lines were obtained until soluble complexes were present in the supernatant in antigen excess. The strongest lines were present in the region of two to five times antigen excess where the largest complexes were formed. Beyond twenty times antigen excess, no precipitation occurred. No precipitation occurred with either antigen or antisera alone.

### DETECTION OF γ-GLOBULIN COMPLEXES IN PATHOLOGICAL SERA AND JOINT FLUIDS

Winchester, Agnello and Kunkel (1969) have recently reported studies on γ-globulin complexes in joint fluids of patients with rheumatoid arthritis which are associated with low complement levels. The complexes were initially demonstrated by ultracentrifugation

FIG. 5. Precipitation of C1q with joint fluids from patients with rheumatoid arthritis. A, purified C1q; 1, joint fluid W.E.; 2, joint fluid M.H.; 3, joint fluid M.Y.; 4, joint fluid J.M.

experiments, precipitation with isolated rheumatoid factor, and by inhibition of rheumatoid factor tests. However, precipitation with C1q also proved useful for their detection (Winchester *et al.*, 1969; Winchester, Agnello and Kunkel, 1970). Fig. 5 shows the reaction of joint fluids M.H. and W.E. which contained large amounts of $\gamma$-globulin complexes as determined by ultracentrifugation and other methods. Joint fluids J.M. and M.Y. with no detectable complexes by other methods gave no reaction with C1q. Reduction and alkylation of the reactive joint fluids eliminated their ability to precipitate with C1q. Interfering precipitation reactions can be given by large amounts of DNA in some joint fluids but this is readily distinguishable by reduction and alkylation and DNAase digestion of the joint fluid. The sera of patients M.H. and W.E., in contrast to their joint fluids, gave no precipitation with C1q.

Certain hypocomplementaemic sera of patients with systemic lupus erythematosus (SLE) were also found to precipitate with C1q in gel diffusion (Agnello, Winchester and Kunkel, 1969). An example is given in Fig. 6 which shows the serial study of sera from patient S.V. The precipitation of C1q with heat-aggregated $\gamma$-globulin is shown for comparison. The precipitin reaction which was weak in January (1) became stronger with increased disease activity in April through July. Following treatment with high dose steroids, the precipitin line disappeared in September.

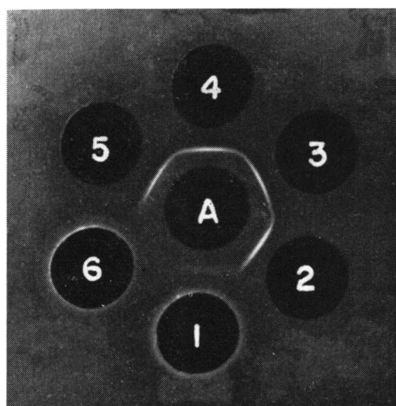


FIG. 6. Gel precipitation of C1q (well A) with serial sera from an SLE patient followed over a period of 8 months in 1968. 1, 28th January; 3, 30th April; 4, 17th May; 5, 20th July; 6, 19th September. The control reaction with aggregated F11 (well 2) is also shown.

TABLE 3

C1q PRECIPITATION WITH VARIOUS HUMAN SERA

| | No. tested | No. positive |
|---|---|---|
| Hypocomplementaemic SLE | 17 | 11 |
| Normocomplementaemic SLE | 41 | 0 |
| Hypocomplementaemic chronic glomerulonephritis | 8 | 0 |
| Rheumatoid arthritis | 30 | 0 |
| Miscellaneous hypocomplementaemic disease | 8 | 2 |
| Hospital patients | 125 | 2 |

Similar precipitation of C1q was found with eleven of seventeen sera from SLE patients that had active disease and hypocomplementaemia. Seven were available for serial study and in all the precipitin reaction disappeared with clinical improvement and a rise in serum complement. Nephritis was present in seven of the reactors. The number of reactors found among sera from normocomplementaemic SLE patients and other types of sera is shown in Table 3. The two positive 'hypocomplementaemic disease' sera were from a patient with chronic haemolytic anaemia and another with erythema multiforme and oedema of unknown aetiology. The two positive 'hospital' sera were weak reactions which were not eliminated by reduction and alkylation of the sera; both patients had sepsis. The serum complement levels were normal.

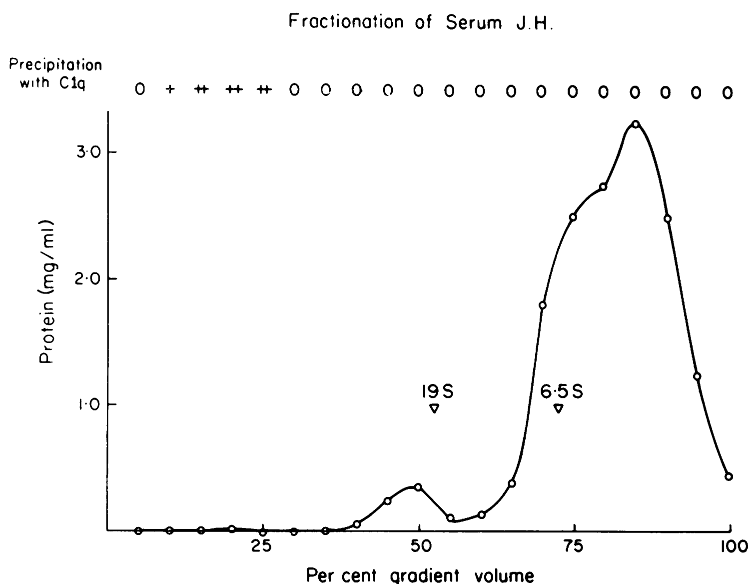


FIG. 7. Sucrose density gradient fractionation of a hypocomplementaemic SLE serum. Precipitation of gradient fractions with C1q in gel diffusion is indicated at top of figure.

Density gradient ultracentrifugation of two of the reactive sera showed that high molecular weight material is involved in the precipitation with C1q (Fig. 7). The 19S and 6·5S markers are indicated by arrows. The C1q precipitable complexes are found in the bottom 25 per cent of the gradient volume and are considerably heavier than 19S. DNAase or RNAase treatment of the serum did effect the reaction with C1q; however, it was eliminated by reduction and alkylation of the serum.

The precipitin curve of one SLE serum, G.G., with C1q is shown in Fig. 8. Such precipitated complexes could be dissociated in 6 M urea and pH 2·5 glycine buffer and analysed further. Preliminary studies employing analytical and density gradient ultracentrifugation have shown the dissociated complexes to consist predominantly of γG globulin. Lesser amounts of C1q, trace amounts of γM and a considerable amount of heavy material which has not been identified were also present. The γG globulin which comprises 60–70 per cent of the total protein consists mainly of γG1 and γG3 subgroups. The isolated γG globulin showed no tendency to aggregate and did not react with C1q. Further studies are under way to elucidate the exact nature of the complexes in these sera.

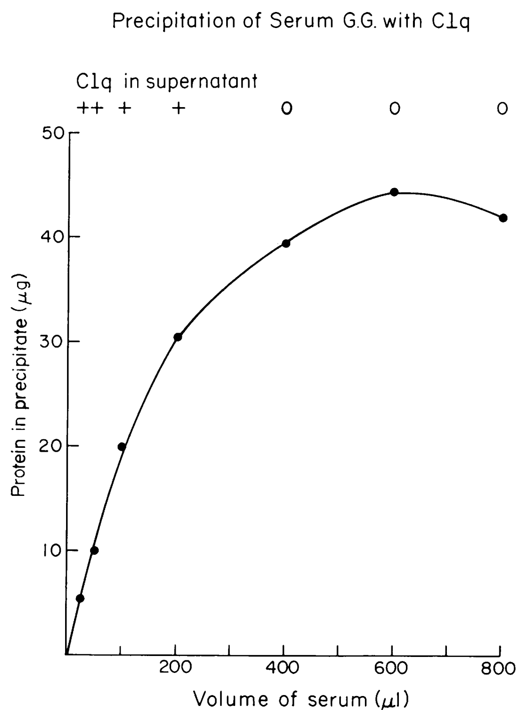


FIG. 8. Curve of precipitation between varying amount of a hypocomplementaemic SLE serum and a constant amount of isolated C1q (20 μg).

## DISCUSSION

Prior studies have not utilized gel diffusion methods to investigate the reaction of C1q with aggregated γ-globulin and immune complexes although the precipitation of immune complexes by C1q in agar gel is known to interfere with interpretations of antigen–antibody reactions (Paul and Benacerraf, 1965). In the present investigations, the C1q in normal human serum readily formed precipitin lines with aggregated γ-globulin and artificially formed immune complexes. These were most evident after addition to EDTA to the serum. Somewhat stronger lines formed with isolated C1q and this was employed in most experiments. Isolation of C1q by precipitation with DNA proved to be a simple

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and useful procedure which utilized the unique cationic properties of this serum protein. The gel reactions occurred best at low concentrations of agarose and optimal conditions of pH and ionic strength for precipitation were worked out. These were relatively close to physiological conditions and in general paralleled those for precipitation of C1q in solution. Analyses of the size of the aggregates and immune complexes precipitating with C1q in the gel system indicated that best precipitation was obtained with larger sizes as noted previously for complement fixation (Osler, 1958; Ishizaka, Ishizaka and Banovitz, 1965; Linscott, 1969). No precipitation occurred with monomeric γ-globulin of any of the γ-globulin subgroups. However, it is of interest that inhibition of the aggregate reactions could be demonstrated in the gel with large amounts of aggregate free normal γ-globulin or isolated myeloma proteins.

The gel procedure proved of particular utility for screening sera and other body fluids for the presence of possible immune complexes of unknown character. Positive sera and joint fluids were found primarily where complement levels were low and other evidence for interactions involving the complement system was evident. The exact nature of the material in SLE sera which precipitates with C1q remains unknown. However, since similar precipitates were obtained in solution, the active material could be concentrated and isolated from the precipitates after solubilization in acid. γG globulin represented the primary constitutent but other unidentified materials are also present which could possibly represent antigens of immune complexes. These questions should be answerable and extensive studies on the dissolved precipitates are under way. Somewhat more information is avilable on the complexes in the joint fluids of patients with rheumatoid arthritis. Here anti γ-globulins of the γG type represent a major constituent of the material which precipitates with C1q (Winchester *et al.*, 1969; Winchester *et al.*, 1970). The active material in SLE sera is clearly different in size and composition and lacks all such anti γ-globulins. It would appear that these materials precipitating with C1q are also involved in the *in vivo* complement consumption. Their presence was clearly related to hypocomplementaemia. Little is known about the exact mechanism of complement depletion in different diseases. Differences have been encountered in the patterns of depletion with respect to individual components in different types of glomerulonephritis (West, Northway and Davis, 1964; Gewurz, Pickering, Mergenhagen and Good, 1968; Kohler and Bensel, 1969). It seems probable that multiple factors are involved and deposition of complement in the glomerulus represents only one part of the picture. It remains unclear whether the complexes detected in the SLE sera play a significant role in the renal disease. They do not appear to be related to the DNA–anti DNA system which represents the primary type thus far implicated in SLE glomerulonephritis (Koffler, Schur and Kunkel, 1967).

Neither the precipitin reaction with C1q in gels nor in solution differentiates reactions due to immune complexes from those due to non specifically aggregated γ-globulin. The latter type did not appear to be involved in the serum or joint fluid reactions described above. However, some old sera were encountered where such aggregated γ-globulin probably was responsible for some weak reactions. In addition certain γG3 myeloma proteins which were known to demonstrate concentration dependent aggregation (Capra and Kunkel, 1970) gave precipitin reactions in the gels. Polyanions such as DNA sulphate canal so give false positive reactions. These can be differentiated readily by their persistence after reduction and alkylation in contrast to the reactive materials containing γ-globulin. In view of these problems, it is essential that the gel method be used in conjunction with other procedures that help define some of the properties of the active substance involved.

## ACKNOWLEDGMENTS

We should like to thank Dr Charles L. Christian, Dr Raymond L. Sherman and Dr Madoka Shibuya for supplying various sera used in this study and Mrs Paul Handsman, Miss Linda Hoffman and Miss Elena Huang for excellent technical assistance. This investigation was supported by the Public Health Service Grant AM-04761.

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