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C1q Protein of Human Complement†

Mary Ann Calcott and Hans J. Müller-Eberhard*

ABSTRACT: C1q is a subcomponent of the first component of complement which exhibits affinity for various immunoglobulins. It is an unusual protein in that, as a normal plasma constituent, its chemical composition bears resemblance to that of collagen. A method of isolation of C1q from human serum has been described which affords preparation of the protein in a high degree of ultracentrifugal and immunochemical homogeneity. Purification was 370-fold and the yield ranged between 3 and 12% of the amount of C1q in serum. The molecular weight of C1q was 393,000 or 410,000, depending on the method of determination. Evidence for dissociation into noncovalently linked subunits was obtained by electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulfate. C1q contains 181 residues of glycine, 17 residues of hydroxylysine, and 51 residues of hydroxyproline per 1000 amino acid residues. The total carbohydrate content of the protein is 9.8% by weight, 6.45% by weight consisting of approximately equimolar amounts of galactose and glucose. Evidence was obtained suggesting that a glucose–galactose moiety is linked to hydroxylysine.

The subcomponent C1q[1] represents one of three different proteins occurring in plasma as a calcium-dependent complex which constitutes the first component of complement (Lepow *et al.*, 1963). C1q is able to distinguish between various immunoglobulins in that it enters into protein–protein interaction with γG and γM, but not with γA, γD, and γE. One molecule of C1q carries five to six binding sites for γG. Interaction of C1q with immune complexes results in activation of the complement system. These antibody-like properties prompted a chemical investigation of the protein. An initial chemical exploration showed the presence of hydroxylysine

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[1] The nomenclature of complement used is that recommended by the World Health Organization (*Bull. WHO* (1968), *39*, 935). The components of complement (C) are designated numerically, C1–C9. The subcomponents of C1 are called C1q, C1r, and C1s. Intermediate reaction products of complement-dependent cytolysis consisting of cells (E), antibody to cell surface antigens (A), and complement components are symbolized by notations which indicate the components required for their formation: EAC1; EAC1,4; EAC1,4,2, etc.

and a large amount of glycine (Müller-Eberhard, 1969). Then hydroxyproline was detected and protein-bound glucose was found which does not commonly occur in the carbohydrate moiety of plasma proteins (Müller-Eberhard, 1971). A previously described method of isolation utilized the affinity of C1q for aggregated γ-globulin (Müller-Eberhard and Kunkel, 1961). To circumvent the use of a biological reagent, a method was devised based solely on standard protein separation techniques. The purpose of this paper is to report our present method of isolation and to relate in detail the unusual properties of C1q which resemble some of the characteristics of collagen.

Materials and Methods

Serum and Serum Fractions. Fresh serum was obtained from healthy blood donors. The euglobulin fraction of 850 ml of serum was prepared by adjusting the pH to 7.0 with glacial acetic acid at 4° and then adding three volumes of cold distilled water to reach a conductance of 2.5 mmhos/cm (all conductance measurements were performed at 22°). After stirring for 30 min at 4°, the developing precipitate was sedimented by centrifugation at 1100*g* and 4° for 30 min. The precipitate was washed with cold sodium phosphate buffer (pH 7, 0.01 M), adjusted to 2.5 mmhos/cm. The precipitate was dissolved in 8 ml of starting buffer for CM-cellulose chromatography (see below), 300 mg of NaCl being added to increase the solubility of the euglobulins. The protein solution was then subjected to ultracentrifugation in a Spinco rotor no. 40 at 35,000 rpm and 4° for 30 min, and the cleared solution was recovered by puncturing the bottom of the centrifuge tube, leaving behind the flotated lipids. Before chromatography the solution was dialyzed against 1 l. of chromatography starting buffer for 1 hr to remove excess NaCl. Chloramphenicol (Parke-Davis, Ann Arbor, Mich.) and kanamycin (Bristol Laboratories, Syracuse, N. Y.) were added as preservatives to all buffers, except the electrophoresis buffer (0.8 ml of a 2% solution of chloramphenicol in ethanol and 0.8 ml of a 2% solution of kanamycin in water per l. of buffer).

CM-cellulose Chromatography. The euglobulin fraction was applied to a 4.5 × 60 cm column containing 700 ml of packed CM-cellulose equilibrated with 0.2 M sodium phosphate buffer (pH 5), containing 0.001 M EDTA (starting buffer). The protein was eluted stepwise, first with starting buffer containing 0.09 M NaCl which was adjusted to a conductance of 19 mmhos/cm. After 1200 ml of effluent was collected in fractions of 20 ml, the buffer was removed from the top of the column and elution was continued with 1200 ml of starting buffer containing 1.4 M NaCl and having a conductance of 105 mmhos/cm.

Gel Filtration. The pool of active material from the CM column (approximately 100 ml) was concentrated by precipitation with ammonium sulfate, using 28 g/100 ml. After 5 hr at 4° the precipitate was collected by centrifugation at 670*g* for 30 min at 4° and the supernatant was removed by syphon. The wet precipitate was transferred into $^7/_{16}$-in. Visking dialysis tubing and dialyzed against 2 l. of 0.28 M sodium phosphate buffer (pH 5.3), until the protein was dissolved. This solution, in a maximum volume of 8 ml, was centrifuged at 670*g* for 5 min to remove undissolved material and applied to a 5 × 100 cm column containing 1300 ml of packed Sephadex G-200 equilibrated with the same buffer. The flow rate was adjusted to 27 ml/hr and 8-ml fractions were collected.

Preparative Electrophoresis. The active material obtained from the Sephadex column was precipitated with ammonium sulfate as described above, dissolved in 5 ml of the electrophoresis buffer, and dialyzed overnight. The protein was applied to a 1 × 18 × 50 cm block of Pevikon C-870 (Müller-Eberhard, 1960) in sodium phosphate buffer (pH 6), ionic strength 0.1, with the application made 20 cm from the anodal end. Electrophoresis was carried out for 40 hr at a potential gradient of 4 V/cm. The block was then cut into 1.25-cm wide segments and each segment was eluted twice with 7 ml of phosphate buffer (pH 6), ionic strength 0.2. The active fractions were pooled and the protein concentrated by ammonium sulfate precipitation, as described above. The precipitate was suspended in 2 ml of 0.05 M sodium phosphate buffer (pH 7) and dialyzed against the same buffer overnight. Concentration by ultrafiltration was avoided since this procedure resulted in very poor yields of C1q protein.

Molecular Weight Determination. The molecular weight of C1q was calculated from sedimentation and diffusion data. Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge at 52,640 rpm and 20° in 0.05 M sodium phosphate buffer (pH 7). Diffusion coefficients were estimated according to Andrews (1965) using a 1.8 × 100 cm column filled with Sagarose 6 or Sagarose 8 (Seravac Laboratories Ltd., Maidenhead, England) in 0.05 M sodium phosphate buffer (pH 7) with the flow rate adjusted to 7 ml/hr. Purified C1q (0.5 ml of 2 mg/ml) was applied to the column and 0.5-ml fractions were collected. Reference substances used were [^{125}I]thyroglobulin ($D = 2.5 \times 10^{-7}$ cm^2/sec), [^{131}I]γG-globulin ($D = 3.8 \times 10^{-7}$ cm^2/sec), human hemoglobin ($D = 6.8 \times 10^{-7}$ cm^2/sec), and equine cytochrome *c* ($D = 13 \times 10^{-7}$ cm^2/sec).

The molecular weight of C1q was also determined according to Archibald by approach to sedimentation equilibrium using the method described by Trautman and Crampton (1959). Analysis was carried out in 0.05 M sodium phosphate buffer (pH 7) at a protein concentration of 4.05 mg/ml at 8766 rpm and 20°. The molecular weight of C1q subunits was estimated by sodium dodecyl sulfate gel electrophoresis performed according to Shapiro *et al.* (1967). C1q was treated with 1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.2), containing 0.001 M iodoacetic acid for 2 hr at 37° and dialyzed overnight at 20° against 0.1% sodium dodecyl sulfate in the same buffer. Reference substances used for molecular weight determination were pepsin (34,000), egg albumin (43,000), bovine serum albumin (68,000), and equine transferrin (74,000). Treatment with 0.01 M dithiothreitol was carried out in 0.55 M Tris buffer (pH 8.5) for 1 hr at room temperature and the protein was then dialyzed against 0.1 M phosphate buffer (pH 7.2), containing 0.01 M iodoacetic acid. It was then treated with sodium dodecyl sulfate as described above.

Chemical Analyses. Protein determinations were made using the Folin method (Lowry *et al.*, 1951) which was standardized with a preparation of highly purified C1q. Nitrogen determination was performed on C1q which was thoroughly dialyzed against distilled water, lyophilized, and dried in a desiccator over $CaCl_2$ at 50° for 48 hr. Neutral hexose was determined by the anthrone method (Mokrasch, 1954), using as standard an equimolar solution of galactose and glucose. Hexosamine was quantitated after 5-hr hydrolysis in 4 N HCl at 105° using the amino acid analyzer. Neuraminic acid was determined by the thiobarbituric acid method (Warren, 1959). C1q-bound glucose and galactose were assayed after 4-hr hydrolysis in 2 N H_2SO_4 at 105° using the glucostat

and galactostat reagents (Worthington Biochemical Corp., Freehold, N. J.). The hydrolysate was neutralized with barium hydroxide and the barium sulfate precipitate was removed by centrifugation. The neutralized hydrolysate was lyophilized and dissolved in 0.15 M potassium phosphate buffer (pH 7).

For paper chromatography of the neutral carbohydrate constituents, a 1 mg-equiv of lyophilized C1q hydrolysate was taken up in 0.05 ml of 50% ethanol. The sample was applied to a 20 × 55 cm strip of Whatman No. 1 filter paper and subjected to descending chromatography for 96 hr at 20° using as solvent 1-butanol–ethanol–water (10:1:2). The dried paper was stained with the silver stain method of Trevelyan *et al.* (1950). As standard a mixture of galactose, glucose, mannose, and fucose (2%, w/v, each) was employed. In several experiments the carbohydrates were eluted from the paper for quantitation following the procedure described by Spiro (1966).

Preliminary characterization of the C1q carbohydrate moiety in alkaline hydrolysates was performed as follows. C1q (10 mg) was hydrolyzed in 1 ml of 2 N NaOH in a polypropylene tube for 24 hr at 105°. After addition of 1 ml of 2 N HCl, the sample was applied to a 1 × 90 cm column of Sephadex G-25 equilibrated with 0.1 M pyridine–acetic acid buffer (pH 5). Fractions (1 ml) were collected and analyzed for peptide and carbohydrate using the ninhydrin and anthrone reagents. Anthrone-positive fractions were pooled and lyophilized, and the material was dissolved in 1.5 ml of 2 N H_2SO_4 and subjected to hydrolysis for 4 hr at 105° and neutralized with barium hydroxide. An aliquot was used for quantitation of hydroxylysine by automatic amino acid analysis, a second aliquot was used for qualitative, and a third aliquot for quantitative paper chromatography of neutral carbohydrates.

Amino acid analyses were made with a Beckman Model 120C analyzer. Protein (1 nmole) was hydrolyzed in redistilled 6 N HCl at 105° for 18 hr (Spackman *et al.*, 1958). Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, following oxidation of the protein with performic acid (Hirs, 1956). For identification of the unknown ninhydrin-positive material which was eluted near the theoretical position of tryptophan, acid hydrolysis was carried out for 72 hr and the chromatographic elution and the absorption characteristics were compared to a hydroxylysine standard (Calbiochem, Los Angeles, Calif.).

For detection and quantitation of hydroxyproline, chromatography on the neutral and acidic residues column of the amino acid analyzer was performed in 0.2 N sodium citrate buffer (pH 3.20) at 32°. For qualitative detection of hydroxyproline in hydrolysates of C1q, samples were subjected to two-dimensional ascending paper chromatography using as solvents *tert*-butyl alcohol–water–formic acid (solvent I) and phenol–water–NH_4OH (solvent II) (Rockland and Underwood, 1965). The paper was stained with isatin reagent (Bailey, 1967). The position of the blue staining spots was compared to that of proline and hydroxyproline standards chromatographed under identical conditions.

Immunochemical Methods. Specific antisera to C1q were produced in rabbits by injection of 100 μg of purified protein in complete Freund's adjuvant into the popliteal lymph nodes. Four weeks after the first injection, the animals were given one injection of 200 μg of C1q in complete Freund's adjuvant intramuscularly. After 1 week the animals were bled and the antiserum was adsorbed with Cohn fraction II γ-globulin to remove any antibody to γG-globulin. Immunochemical quantitation of C1q in serum and serum fractions was performed by the single radial immunodiffusion technique standardized with highly purified C1q protein (Kohler and Müller-Eberhard, 1969).

Immunochemical purity of isolated C1q was tested by the double diffusion-in-gel method using potent monospecific antisera to IgG, IgA, and IgM (Behringwerke A. G., Marburg/Lahn, Germany), and antisera to human C1s, C3, C4, and C5 which were prepared in this laboratory. The gels were prepared with 0.6% agarose (Marine Colloid, Inc., Springfield, N. J.) in sodium phosphate buffer (pH 7.2), ionic strength 0.1 containing 0.01 M Na_3EDTA and 0.05% sodium azide. Immunoelectrophoresis was performed for 2 hr at 20° using 0.5% agar in phosphate buffer (pH 7), ionic strength 0.1, and a potential gradient of 5 V/cm.

Hemolytic Activity Measurements. C1q hemolytic activity during purification was detected semiquantitatively using a human serum reagent selectively depleted of C1q (Müller-Eberhard and Kunkel, 1961).

Quantitative hemolytic titrations were performed on the final purified product using the following procedure. Amounts of C1q ranging from 1 to 100 ng in 0.4 ml of buffer (see below) were incubated for 15 min at 37° with 0.2 ml of a preparation of partially purified C1r and C1s (Lepow *et al.*, 1963), following which 0.2 ml of EAC4 (a complex of sheep erythrocytes, antibody to sheep erythrocytes, and the fourth component of human complement) was added and the mixture was incubated for 30 min at 37°. The number of EAC4 per 0.2 ml was 3×10^7 and the number of C4 molecules bound per cell was approximately 3000 (Cooper *et al.*, 1970). Low ionic strength (0.065) Veronal–NaCl buffer was used which was rendered isotonic with sucrose (Rapp and Borsos, 1963) and which contained 0.1% gelatin. The resulting EAC1,4 intermediate complex was washed twice and suspended in 0.2 ml of the same buffer. Oxidized, isolated human C2 (Polley and Müller-Eberhard, 1967, 1968) was then added (1 μg/0.2 ml) and incubated for 10 min at 32°. The EAC1,4^{oxy}, 2 complex was then treated with 2.4 ml of guinea pig serum diluted 1:100 with Veronal–NaCl buffer containing 0.04 M EDTA. After 60 min at 37° the unlysed cells were removed by centrifugation and the hemoglobin content of the supernatant was measured spectrophotometrically at 415 nm. The data were evaluated in terms of effective molecules according to Mayer (1961).

Results

Isolation of C1q. Since C1q is relatively insoluble at low ionic strength, the first step of the isolation procedure consisted of precipitation of the euglobulins from fresh human serum. The precipitate was dissolved in 0.2 M sodium phosphate buffer (pH 5) and was then submitted to chromatography on CM-cellulose under conditions which retained C1q but allowed removal of 80% of the euglobulins (Figure 1). C1q together with some other proteins was eluted by a stepwise elution procedure and the fractions containing C1q activity were pooled and concentrated by precipitation of the protein with ammonium sulfate. The precipitate was dissolved by dialysis against 0.28 M sodium phosphate buffer (pH 5.3) and subjected to filtration on a Sephadex G-200 column (Figure 2). C1q was eluted with the exclusion volume and was separated from proteins with a lower molecular weight, primarily IgG. The C1q-containing fractions were pooled and the protein was concentrated by precipitation with ammonium sulfate. It was dissolved in electrophoresis buffer and subjected to Pevikon block electrophoresis at pH 6.

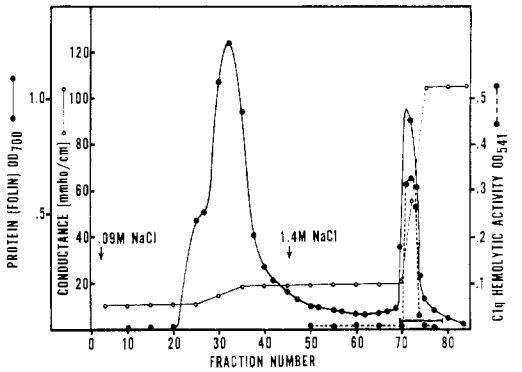

FIGURE 1: Second step of Clq isolation: chromatography of human serum euglobulins on CM-cellulose. The protein obtained in the first step of the procedure was eluted, using 0.2 M sodium phosphate buffer (pH 5), containing 0.001 M EDTA and respectively 0.09 and 1.4 M NaCl as indicated. Clq hemolytic activity was eluted together with the final protein peak at a conductance above 22 mmhos/cm. Fractions were pooled as indicated by the horizontal bar.

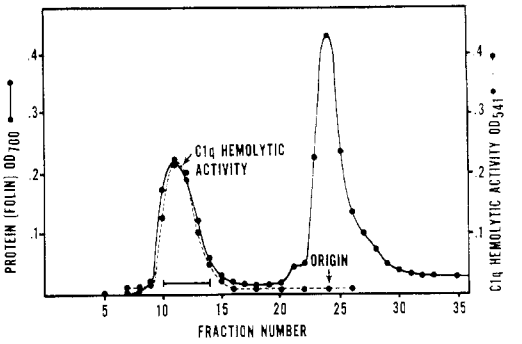

FIGURE 3: Fourth step of Clq isolation: preparative electrophoresis on Pevikon block. The concentrated protein contained in the exclusion volume from the Sephadex column was subjected to electrophoresis in sodium phosphate buffer (pH 6), ionic strength 0.1, for 40 hr at 4 V/cm and 4°. The Clq hemolytic activity corresponds to the protein which migrated toward the cathode. The anode was at the right and fractions were pooled as shown by the bar.

This procedure allowed separation of the basic C1q protein from contaminating serum constituents with more negative charge, *i.e.*, IgM, some C3 and C5 (Figure 3). The cathodally migrating protein correlated in distribution with the hemolytic activity of C1q. Fractions were pooled as indicated in Figure 3, concentrated by ammonium sulfate precipitation and dialyzed against phosphate buffer (pH 7), ionic strength 0.1, for further analysis.

Table I shows the recovery of C1q protein from the consecutive steps of the isolation procedure for preparation 83. In serum, C1q represents 0.28% of the total protein present. The final yield was 0.016% of total serum protein, or 5–6% of total serum C1q. This represents a 370-fold purification. Of 105 different preparations, the yield ranged from 3.3 to 12%.

Physical and Immunochemical Homogeneity of Isolated C1q. Figure 4 shows the ultracentrifugal homogeneity of isolated C1q. The pattern depicted is representative for most preparations obtained. In a few instances, some heavier or lighter material was observed which, however, accounted for less than 5%. Electrophoretic homogeneity of the protein at pH 6 is indicated by its behavior on Pevikon blocks during the last preparative step, as shown in Figure 3. The immunochemical examination of C1q by Ouchterlony's double-diffusion-in-gel technique is depicted in Figure 5. It gave negative reactions with antisera to IgG, IgA, and IgM and strong, positive reaction with an anti-C1q. Occasionally, a weak reaction with anti-IgG was seen, however, comparative analyses to known amounts of IgG indicated that contamination of C1q with IgG did not exceed 1% w/w. Negative reactions were also obtained with antisera to various β-globulins, such as C1s, C3, C4, and C5.

Characterization of C1q. The sedimentation coefficient of C1q was previously found to be $s^0_{20,w} = 11.1$ S (Müller-Eberhard and Kunkel, 1961). The diffusion coefficient was estimated according to Andrews using a Sagarose 8 column and thyroglobulin, IgG, hemoglobin, and cytochrome *c* as reference substances. As shown in Figure 6 the behavior of C1q was very similar to that of thyroglobulin, $D = 2.5 \times 10^{-7}$ cm²/sec. The molecular weight calculated from *s* and *D* and an assumed partial specific volume of 0.73 was 393,000. The molecular weight was also determined by the Archibald method. By the least-squares method the resulting $c_m - c^0$ *vs.* q_m plot was represented by $y = -109{,}387 \times +44{,}094.2$. The molecular weight was 410,000. The frictional ratio obtained from a nomogram (Wyman and Ingalls, 1943) was 1.7.

The immunoelectrophoretic analysis of isolated C1q is

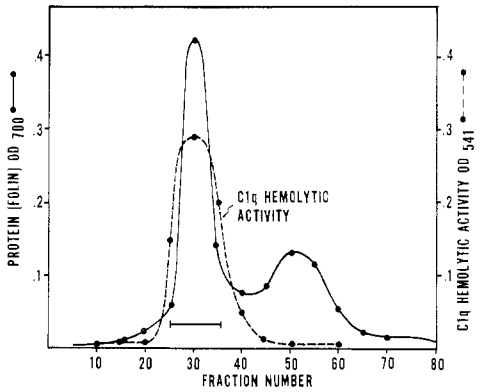

FIGURE 2: Third step of Clq isolation: gel filtration on Sephadex G-200. The concentrated, Clq-containing material from CM-cellulose chromatography was eluted with 0.28 M sodium phosphate buffer (pH 5.3). Clq hemolytic activity was eluted with the protein fraction of the exclusion volume, and Clq-containing fractions were pooled as indicated by the bar.

TABLE I: Recovery of C1q during Isolation Procedure.

Material	Vol (ml)	Total Protein[b] (mg)	C1q[b] (mg)	% C1q (w/w)
Serum	800	56,000	152	0.28
Euglobulin	40	1,470	59	4.00
CM effluent pool	255	178	48	27.00
Sephadex effluent pool	213	54	28	52.00
Pevikon block pool	95	11	13	118.00
$(NH_4)_2SO_4$ precipitate	3	9	8	89.00
Yield (%)		0.016	5.3	

[a] By Folin method. [b] By immunochemical assay.

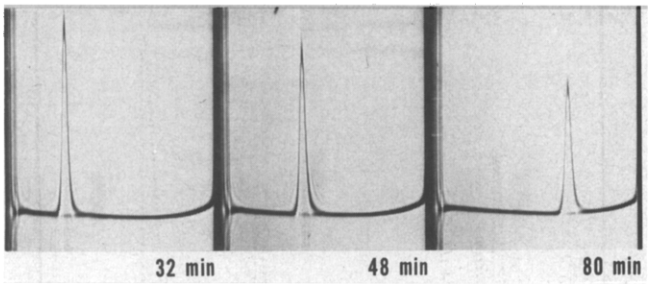

FIGURE 4: Ultracentrifugal analysis of isolated Clq. The protein was dissolved in 0.05 M sodium phosphate buffer (pH 7) and the concentration was 3.8 mg/ml. The speed was 52,640 rpm, temperature 20° and the phase-angle 55°. The photographs of the schlieren patterns were taken at the indicated time intervals.

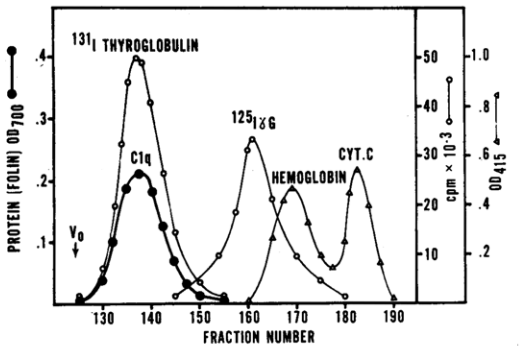

FIGURE 6: Estimation of the diffusion coefficient of isolated Clq: gel filtration on Sagarose 8. The column size was 1.8 × 100 cm, the buffer was 0.05 M sodium phosphate (pH 7), the flow rate 7 ml/hr and the fraction volume 0.5 ml. Clq (2 mg) was applied and detected in the effluent by the Folin method. The reference substances were applied in trace amounts and thyroglobulin and γG were detected by radioactivity determination and hemoglobin and cytochrome *c* spectrophotometrically at 415 nm.

depicted in Figure 7 and compared with the pattern of the β- and γ-globulins of human serum. At pH 7, C1q migrated further toward the cathode than IgG.

The nitrogen content was found to be 12.9% (w/w). Amino acid analysis was performed on ten different preparations and representative results are listed in Table II. To exclude the possibility that the high glycine concentration was caused by noncovalently bound glycine, C1q was examined after dialysis for 16 hr against 5 M urea followed by dialysis against sodium phosphate buffer (pH 7) and after precipitation with 10% trichloroacetic acid. In both instances, the glycine content was 181 residues/1000 amino acid residues. In addition to the amino acids known to occur in plasma proteins, ninhydrin-positive material was eluted from the basic residues column of the amino acid analyzer between glucosamine and lysine. This material was identified as hydroxylysine on the basis of its position in the chromatogram and the colorimetric characteristics of its ninhydrin reaction product (ratio of absorbance at 570 and 440 nm). Figure 8 shows comparable segments of the chromatograms of a standard amino acid and hexosamine mixture and of a hydrolysate of C1q. Hydroxylysine of the standard solution was eluted exactly 45 min after sample injection, corresponding to the unknown material in the C1q hydrolysate. Examination of the $A_{570}:A_{440}$ ratio of the aspartic acid peak in the C1q chromatogram showed it to be lower than that of aspartic acid of the standard solution, suggesting the presence of hydroxyproline. Chromatography of the hydrolysates of two different C1q preparations was performed on the column for acidic and neutral amino acid residues under conditions allowing separation of hydroxyproline and

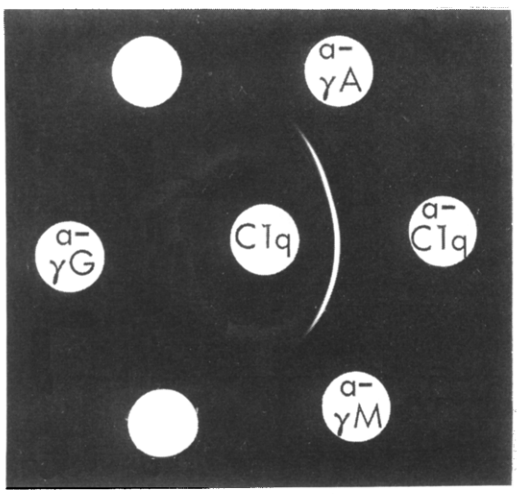

FIGURE 5: Immunochemical analysis of isolated Clq. Clq was examined at a concentration of 250 μg/ml by the double-diffusion-in-gel technique with antisera to human Clq, γG, γA, and γM. Clq did not react with any of the antisera to immunoglobulins. That the Clq–anti-Clq precipitin line is concave toward the antigen well is consistent with the high molecular weight of the antigen. (The undesignated wells were empty.)

TABLE II: Amino Acid Composition of C1q.

	Amino Acid Residues			
	g/100 g	Mole/1000 Moles	No./ Molecule[a]	No./ Peptide Moiety[b]
Hydroxy-proline	5.42	50.70	196.50	177.19
Aspartic acid	8.19	75.30	291.83	263.16
Threonine	5.04	52.71	204.28	184.21
Serine	4.17	50.70	196.60	177.19
Glutamic acid	11.52	94.38	365.76	329.82
Proline	5.90	64.26	249.03	224.56
Glycine	9.75	180.72	700.39	631.58
Alanine	2.20	43.17	167.31	150.88
Half-cystine[c]	1.52	15.56	60.31	54.38
Valine	5.27	56.22	217.90	196.49
Methionine[c]	2.36	19.07	73.92	66.66
Isoleucine	4.46	41.67	161.48	145.61
Leucine	6.93	64.76	250.97	226.31
Tyrosine	5.19	33.63	130.35	117.54
Phenylalanine	6.35	45.68	177.04	159.65
Hydroxylysine	2.33	17.07	66.15	59.65
Lysine	4.44	36.64	142.02	128.07
Histidine	2.02	15.56	60.31	54.38
Arginine	6.23	42.17	163.42	147.37
Total			3875.47	3494.70

[a] Molecular weight 410,000. [b] Molecular weight minus carbohydrate moiety. [c] Determined as cysteic acid and methionine sulfone.

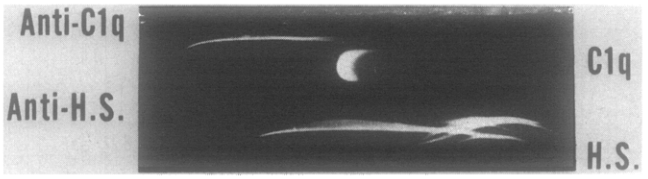

FIGURE 7: Immunoelectrophoretic representation of isolated Clq. Electrophoresis was performed in 0.5% agar in sodium phosphate buffer (pH 7), ionic strength 0.1, for 2 hr at 5 V/cm. For comparison, part of the pattern of whole human serum is shown. Clq assumed a more cathodal position than γG-globulin serum. The anode was to the right.

aspartic acid Figure 9 demonstrates the presence of hydroxyproline in C1q. Two-dimensional paper chromatography followed by staining with an isatin reagent also revealed hydroxyproline to be present.

Carbohydrate analyses were performed on six different preparations of C1q. The average values of the results are listed in Table III. The total carbohydrate content is approximately 9.8%, 6.45% consisting of approximately equimolar amounts of galactose and glucose. Since protein-bound glucose had not been reported for plasma proteins, paper chromatography of the neutral sugars of C1q was performed after mild acid hydrolysis of the protein. As shown in Figure 10, the presence of glucose could be verified in addition to that of galactose and small amounts of mannose and fucose. The molar ratio of hydroxylysine, galactose, and glucose was calculated for whole C1q to be approximately 1 : 1.2 : 1.3.

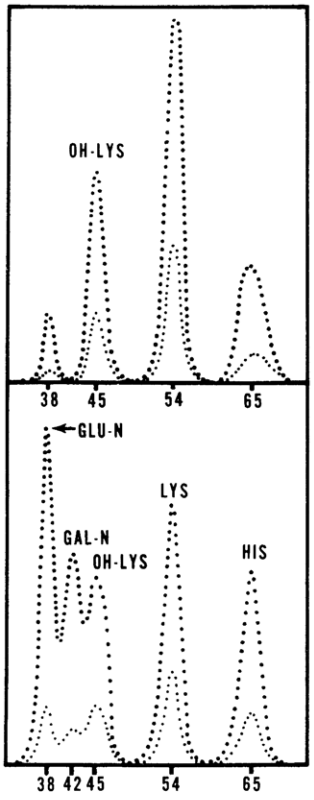

FIGURE 8: Demonstration of hydroxylysine in Clq. The upper panel shows a portion of the chromatogram of the basic amino acids in a 72-hr acid hydrolysate of Clq. The lower panel shows the corresponding portion of the chromatogram of a standard solution of amino acids containing, in addition, glucosamine, galactosamine, and hydroxylysine.

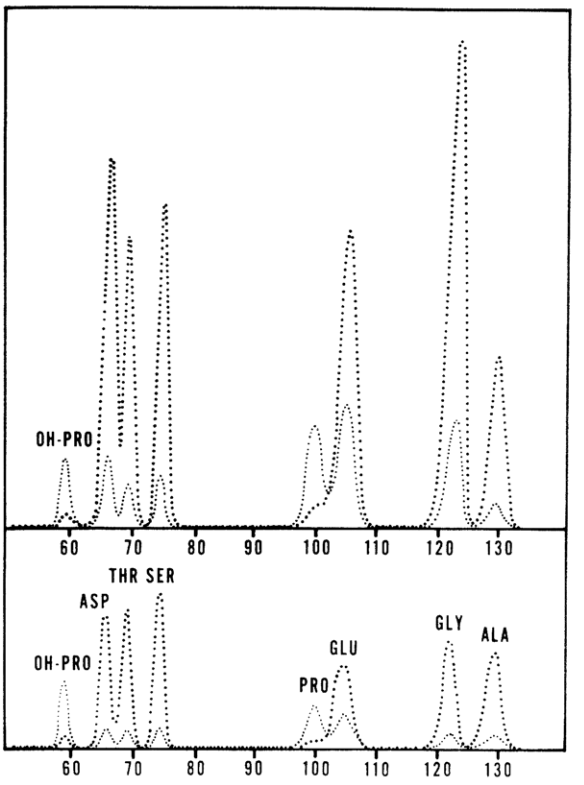

FIGURE 9: Demonstration of hydroxyproline in Clq. The upper panel shows a portion of the chromatogram of the acid and neutral amino acids in an 18-hr acid hydrolysate of Clq. The lower panel shows the corresponding portion of a chromatogram of a standard solution of amino acids, also containing hydroxyproline. Chromatography was performed in 0.2 N sodium citrate buffer (pH 3.20), at 32° to allow separation of hydroxyproline and aspartic acid.

To investigate further the nature of the glucose-containing carbohydrate moiety, an alkaline hydrolysate of C1q was passed over a Sephadex G-25 column in pyridine–acetic acid buffer (pH 5) (Figure 11). The major portion of the anthrone-positive material was eluted before the peak of the ninhydrin-positive substances. A similar elution profile was observed in three separate experiments. The carbohydrate-containing fractions were pooled as indicated in the figure and subjected to mild acid hydrolysis. Paper chromatography of neutral sugars detected galactose and glucose as shown in Figure 10. By quantitative chromatography in a single experiment 96

TABLE III: Carbohydrate Content of C1q.

	% (w/w)
Glucosamine	0.8
Galactosamine	0
Neuraminic acid	0.5
Neutral hexose	8.5
Glucose	3.35
Galactose	3.10
Total	9.8

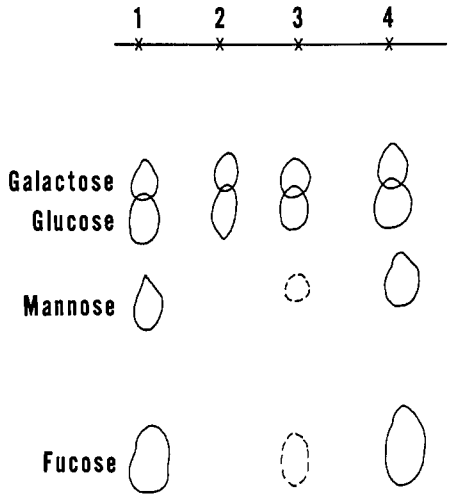

FIGURE 10: Paper chromatography of the neutral carbohydrates of Clq. Samples 1 and 4 are standard mixtures of the indicated carbohydrates. Sample 2 is the anthrone-positive material separated from an alkaline hydrolysate of Clq by Sephadex G-25 (Figure 11), which was subsequently subjected to hydrolysis in 2 N H_2SO_4 for 4 hr at 105°. Sample 3 is a hydrolysate of Clq protein (2 N H_2SO_4, 4 hr at 105°). Descending chromatography was performed for 96 hr at 20° using 1-butanol–ethanol–water (10:1:2). The figure shows a tracing of paper stained with a silver reagent.

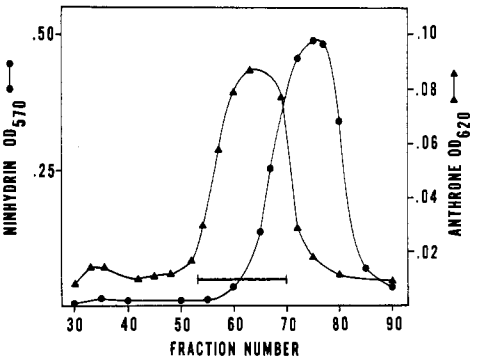

FIGURE 11: Molecular sieve chromatography of the anthrone-positive material in an alkaline hydrolysate of Clq. The alkaline hydrolysate (2 N NaOH, 24 hr, 105°) of Clq was eluted from a Sephadex G-25 column which was equilibrated with 0.1 M pyridine–acetic acid buffer (pH 5). Aliquots used for the anthrone reaction were 0.5 ml and for the ninhydrin reaction 0.02 ml. Fractions were pooled for further analysis as indicated by the bar.

μmoles of galactose and 76 μmoles of glucose were found. The same hydrolysate contained 80 μmoles of hydroxylysine as determined by the amino acid analyzer. These results indicate that the major carbohydrate moiety of C1q consists of similar amounts of galactose and glucose and suggest that these sugars are linked to hydroxylysine.

Hemolytic Activity of Isolated Clq. The hemolytic activity of C1q was assayed by measuring the formation of EAC1,-4 from EAC4 in the presence of C1r, C1s, and Ca^{2+} at ionic strength 0.065. The activity of cell-bound C1 complex was quantitated after thorough washing of the cells with low ionic strength buffer by measuring conversion of EAC1,4 to EAC1,-4,2. Lysis of this intermediate complex is then induced by serum containing 0.04 M Na_3EDTA. Figure 12 shows the quantitative relationship between lysis of sheep erythrocytes and the amount of isolated C1q in the reaction mixture. The extent of lysis was proportional to Clq input.

Dissociation of Clq into Subunits. Treatment of C1q with 0.1 M mercaptoethanol had no effect on its sedimentation coefficient. However, treatment with sodium dodecyl sulfate resulted in dissociation of the protein. By analytical ultracentrifugation the *s* rate in presence of 0.1% sodium dodecyl sulfate decreased from 11 to 3 S. Electrophoresis of C1q in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate revealed a major component with a molecular weight of 65,000–70,000 in addition to several minor protein bands. Upon reduction of C1q with 0.01 M dithiothreitol and subsequent treatment with sodium dodecyl sulfate the 70,000 molecular weight subunit was further dissociated into at least three different subunits with molecular weights below 40,000.

Discussion

The isolation procedure described above has yielded reproducibly highly purified preparations of C1q as judged by ultracentrifugal and immunochemical criteria. The yield of C1q was 3–12% which is similar to that of other complement proteins. Three other methods for the purification of C1q have previously been reported. One method utilized γ-globulin aggregates (Müller-Eberhard and Kunkel, 1961), and another DNA (Agnello *et al.*, 1970) as biological reagents. The third method employs repeated precipitation at low ionic strength (Yonemasu and Stroud, 1971).

Interest in the chemical composition of C1q arose when the protein was found to possess specificity for various immunoglobulins. Ultracentrifugal analysis of mixtures of isolated C1q and native, monomeric γG-globulin disclosed the formation of soluble complexes of the two proteins. In spite of this antibody-like behavior, a relationship to the immunoglobulin system could not be verified. C1q did not react with antisera to human γG, γA, or γM. Unlike immunoglobulins, it was found to contain hydroxylysine, hydroxyproline, and a high concentration of glycine residues. Its sizable carbohydrate moiety (9.8%) contains primarily neutral, anthrone-positive constituents including glucose, which does not occur in immunoglobulins or other serum proteins (Schultze and Heremans, 1966).

The chemical composition of C1q bears resemblance to that of structural proteins. C1q contains 181 glycine, 17 hy-

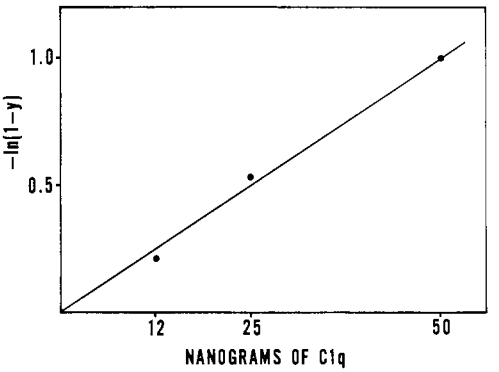

FIGURE 12: Hemolytic activity of isolated Clq. The degree of lysis of sentized sheep erythrocytes, expressed as negative natural logarithm of the fraction of unlysed cells ($-\ln(1-y)$), is plotted as a function of the amount of Clq present in the reaction mixture. *y* represents the fraction of cells lysed. A $-\ln(1-y)$ value of unity corresponds to 63% lysis. The reagents used for the assay supplied all complement proteins except Clq.

droxylysine, and 51 hydroxyproline residues per 1000 amino acid residues. Bovine glomerular basement membrane contains 207 and bovine collagen 312 glycine residues per 1000 residues (Spiro, 1967a; Kefalides and Winzler, 1966). The hydroxylysine content of C1q is virtually identical with that of glomerular basement membrane and approximately three times greater than that of collagen. Its hydroxyproline content is somewhat lower than that of basement membrane (Spiro, 1967a) and half that of bovine collagen (Kefalides and Winzler, 1966). Like these connective tissue proteins, C1q has approximately equimolar amounts of glucose and galactose residues, although its total neutral hexose content is almost twice that of basement membrane (5.85%) and about 15 times higher than the hexose content of collagen (0.6%) (Spiro, 1967a; Kefalides and Winzler, 1966). Similar results were reported by Yonemasu *et al.* (1971) while this study was being concluded and presented (Müller-Eberhard, 1970, 1971).

Butler and Cunningham (1966) showed glucose and galactose of collagen to be linked as disaccharide to the hydroxyl group of hydroxylysine, and Spiro (1967b) isolated from Pronase-degraded basement membrane glucosylgalactosylhydroxylysine. An alkaline hydrolysate of C1q was therefore subjected to molecular sieve chromatography. The major peak of anthrone-positive material preceded ninhydrin-positive substances in the elution profile. Among the anthrone-positive material was found, after mild acid hydrolysis, approximately equimolar amounts of hydroxylysine, galactose, and glucose. This finding is consistent with the assumption that C1q has a carbohydrate moiety analogous to collagen or basement membrane. The small amounts of glucosamine, neuraminic acid, mannose, and fucose detected are indicative of a second carbohydrate moiety resembling in composition that of plasma glycoproteins.

Reduction of the molecular weight of C1q by sodium dodecyl sulfate to 65,000–70,000 suggests that the C1q molecule consists of six noncovalently bonded subunits of similar or identical size. Since the valence of C1q for γG-globulin was found to be 5–6, each of these subunits could carry one immunoglobulin binding site. In view of the relatively large molecular weight of native C1q, we suggested to Dr. S. E. Svehag, Stockholm, to subject the protein to high-resolution electron microscopy. Subsequently, Svehag and Bloth (1970) proposed a compact, disk-like molecular model in which five pentagonal subunits are arranged around a central pentameric unit. Polley (1971) encountered two distinct conformational forms of C1q: one being disk like with a diameter of 200 Å, the other rod like with a length of 400 Å. Shelton *et al.* (1972) have proposed a more elaborate model according to which six-terminal subunits are linked by six connecting strands to one central subunit; the overall diameter of the molecule being 350 Å. Although not completely in agreement with each other, these ultrastructural models are in general agreement with physiocochemically established properties of C1q. Since the noncovalently linked subunits can be further dissociated by disulfide-bond-cleaving reagents into pieces of different size, the question arises as to whether the collagen-like composition is a property of only one type of polypeptide chain. How the unusual chemical characteristics are related to the biological function of C1q remains to be determined.

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