ISOLATION OF β_{1F}-GLOBULIN FROM HUMAN SERUM AND ITS CHARACTERIZATION AS THE FIFTH COMPONENT OF COMPLEMENT*, ‡

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In pursuing work on the classical third component of human complement, a heretofore unrecorded serum protein has been detected and recognized as an essential factor in the later steps of immune hemolysis (1). The purpose of this paper is to describe the procedure of its isolation, some of its molecular properties, and to define the position at which this protein acts in the complement-reaction sequence. On the basis of immunoelectrophoretic characteristics, it has been designated β_{1F}-globulin and in reference to its activity, the fifth component of human complement or C′5.

The existence of β_{1F}-globulin was first discovered by means of antisera prepared to β_{1C}-globulin isolated according to a method described previously (2). While these β_{1C} preparations seemed immunologically homogeneous when analyzed with powerful antisera to whole human serum, they nevertheless gave rise to antibodies to 2 minor contaminants when injected into rabbits; 1 of which was β_{1F}-globulin. The detection of these contaminants necessitated reinvestigation of the role of β_{1C}-globulin in immune hemolysis (3). In the course of this analysis, it was found that the activity previously attributed solely to β_{1C}-globulin is, in fact, the result of at least 3 factors, 1 of which is β_{1C}-globulin, the other β_{1F}-globulin, and the 3rd is a factor which is still not recognized as a discrete protein entity (4). In terms of their activity, these 3 factors will be referred to in the following as the third, fifth, and sixth components of complement, or C′3, C′5, and C′6.

Materials and Methods

Serum and Serum Fractions.—Freshly drawn blood from white donors was purchased from the San Diego Blood Bank, San Diego. After a period of 4 hours at room temperature, during which clotting was allowed to proceed, the serum, approximately 400 ml, was pooled and dialyzed for 18 hours at 4°C against 2 × 10 liters of phosphate buffer, pH 5.4, T/2 = 0.02.

In order to prevent conversion of β_{1C} to β_{1A}, EDTA in a final concentration of 0.005 M was added to the individual sera prior to pooling. By dialysis the EDTA concentration was

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allowed to decrease to 0.0002 M and was maintained at this level until the material was subjected to TEAE-cellulose chromatography.

The euglobulin formed during dialysis was separated from the rest of the serum by centrifugation at 1560 *g* and 2°C for 20 minutes. The precipitate was washed 3 times with 750 ml of dialysis buffer and dissolved in 20 ml of phosphate buffer, pH 8.1, T/2 = 0.03. Some NaCl in substance was added to facilitate solution of the protein. The dissolved euglobulins were separated from lipid material by centrifugation for 1 hour at 20,000 RPM., in a Spinco model L2 ultracentrifuge, using a No. 40 rotor. A clear, faintly yellow-green solution was collected through a hole punched into the bottom of the centrifuge tube. The top layer containing the flotated lipid material was discarded. The euglobulin was dialyzed for 6 to 8 hours against several changes of starting buffer prior to chromatography.

TEAE Chromatography.—Approximately 300 ml of packed TEAE-cellulose in a 2.5 × 60 cm column was equilibrated with phosphate buffer, pH 8.1, T/2, = 0.03 (starting buffer). After the euglobulin was applied, the column was washed with 700 to 900 ml of starting buffer, and elution was then begun by applying a gradient of increasing salt concentration and decreasing pH. An open cone sphere system was used which consisted of a 1000 ml round flask filled with starting buffer and a 500 ml Erlenmeyer flask containing 0.25 M monobasic Na phosphate. Elution was completed with 200 to 300 ml of 0.25 M monobasic phosphate. Fractions containing β_{1C}- and β_{1F}-globulin were concentrated by precipitation in phosphate buffer, pH 5.4, T/2 = 0.02.

Hydroxyl Apatite Chromatography.—OH apatite chromatography was performed according to the procedure described by Tiselius *et al.* (5). Buffers were prepared by combining suitable proportions of 0.02 M Na-phosphate with 0.65 M KNa-phosphate buffer of pH 7.9. The 0.65 M KNa-phosphate buffer was obtained by mixing 0.65 M dibasic potassium phosphate with 0.65 M monobasic sodium phosphate. The different buffers were defined by their conductance at 23°C. Starting buffer had a conductance of 8 mmhos/cm. Approximately 100 ml of packed OH apatite in a 2.5 × 20 cm column were equilibrated with starting buffer. The material obtained by TEAE-cellulose chromatography was then applied to the column without preceding dialysis. After washing of the column with 300 ml of starting buffer, stepwise elution was begun. The conductance of the buffers employed was 12, 13, and 14.5 mmhos/cm, respectively. The column was reused after washing with 0.65 M KNa-phosphate buffer.

Fractions containing β_{1C} were pooled and the protein was precipitated by dialysis against phosphate buffer, pH 5.4, T/2 = 0.02. The precipitate was dissolved in a small volume of phosphate buffer, pH 7, T/2 = 0.1 and NaCl was added to facilitate its solution.

Preparative Electrophoresis.—Fractions containing β_{1F} after OH apatite chromatography were pooled and concentrated by ultrafiltration. The material was then separated by preparative electrophoresis in Pevikon blocks, as previously described (6). The separation was carried out at 4°C, in barbital buffer, pH 8.6, T/2 = 0.05 for 35 hours employing a potential gradient of 3.5 v/cm.

Starch Gel Electrophoresis.—Starch gel electrophoresis was performed according to Smithies (7) using the discontinuous buffer system of Poulik (8). A filter paper carrier was used for application of the samples. Electrophoresis was carried out at 4°C for 12 hours at 5 v/cm. In certain instances protein was eluted with veronal buffer from the unstained half of the gel by freezing and thawing of suitable segments and used for further analysis.

Ultracentrifugation.—Sedimentation coefficients were determined in a Spinco model E ultracentrifuge at 20°C. Phosphate buffer, pH 7, T/2 = 0.1 was used as solvent. Density gradient ultracentrifugation was carried out according to Kunkel (9).

Antisera.—

Anti-β_{1F}: Antibodies to β_{1F} globulin were obtained by immunizing rabbits with purified β_{1F}-globulin or with fractions containing β_{1F}- and β_{1C}-globulin. 3 × 1 mg of protein was given

subcutaneously in complete Freund's adjuvant in weekly intervals. Three weeks after the last injection the animals were bled.

Antisera prepared to a mixture of β_{1C} and β_{1F} were rendered specific for β_{1F}-globulin by adsorption with purified β_{1C}.

Anti-β_{1C}: Specific antisera to β_{1C} were obtained by immunizing rabbits with purified β_{1C}-globulin or with zymosan treated with human serum according to a procedure published elsewhere (10).

Anti-β_{1E}: Rabbit antisera to β_{1E} were prepared by immunization with purified β_{1E}-globulin, as described earlier (11).

Other antisera: Antisera to the following human serum proteins were purchased from Behringwerke AG., West Germany: No. 470E, normal serum; No. 240L, γA-globulin; No. 65, γG-globulin; No. 329A, transferrin; No. 440C, β-lipoprotein; No. 444F, fibrinogen. In addition, a horse anti-γM-globulin was used (No. 477D).

Immunodiffusion techniques: Immunoelectrophoresis was performed according to Scheidegger (12). Immunologic analysis of highly purified β_{1F}-globulin was done on Ouchterlony plates. Localization and semiquantitation of β_{1F} in fractions obtained by chromatography, electrophoresis, or zone ultracentrifugation was carried out on microscopic slides, covered with an agar layer and containing a central longitudinal row of 11 wells and longitudinal lateral troughs. The wells were filled with test fractions and the troughs with antisera.

Diluents, chelating, and bacteriostatic agents: Veronal buffered saline containing Ca^{++} and Mg^{++} (13), with or without 0.1 per cent gelatin (w/v), was used as diluent for complement assays. 0.1 M sodium-ethylenediaminetetraaceticacid (EDTA) solution, pH 7.6, was prepared by mixing 7.44 gm Na_2 EDTA with 7.60 gm Na_4 EDTA in a volume made up to 400 ml with distilled H_2O.

It was noted that bacterial growth sometimes occurred in the chromatography columns, leading to a partial conversion of β_{1C} to a product similar to β_{1A}. This complication was avoided by introducing chloramphenicol as a bacteriostatic agent. A concentration of 5×10^{-5} M was present throughout the separation procedures (0.8 ml of a 2 per cent solution of chloramphenicol in 95 per cent ethanol (w/v) was added per liter of buffer). The high optical density at 280 mμ caused by this concentration of chloramphenicol made OD 280 readings less reliable for protein determinations than the Folin method. Therefore, the latter method was employed in most instances.

Preparation of Complexes Consisting of Erythrocytes, Antibody, and Complement.—

EAC'1a,4,2a: Cells in the state EAC'1a,4,2a were prepared according to the following procedure. A flask containing 7.5×10^9 sensitized sheep erythrocytes in 15 ml veronal buffer was warmed to 37°C. In a second flask 150 mg phloridzin was dissolved in 15 ml veronal buffer by heating to approximately 80°C. Subsequently the temperature was adjusted to 37°C. After the contents of the 2 flasks were mixed, 0.75 ml fresh human serum was added. The mixture was incubated for 2 minutes at 37°C, and was then poured into ice cold gelatin containing veronal buffer. The cells were washed 2 times and resuspended in 15 ml gelatin-veronal buffer, and kept at 0–1°C until used.

EAC'1a,4,2a,3: 10^8 EAC'1a,4,2a cells were treated with 10 μg of purified β_{1C}-globulin for 2.5 minutes at 30°C in a volume of 0.5 ml containing 0.01 M EDTA. The cells were then washed and resuspended to a suitable volume in ice cold gelatin-veronal buffer.

EAC'1a,4,2a,3,5,6: These cells were prepared by incubating 10^8 EAC'1a,4,2a cells for 20 minutes at 37°C in a 0.5 ml volume containing 0.01 M EDTA, 10 μg β_{1C}, 20 μg β_{1F} and a sufficient amount of partially purified C'6. The cells were washed and stored in gelatin-veronal buffer.

C'5 assay: 10^8 EAC'1a,4,2a 0.05 ml 0.1 M EDTA, 10 μg β_{1C}, and a sufficient amount of C'6 were mixed at 0°C with an aliquot of the test material. The amount of C'6 required was de-

termined separately for each new batch of C'6 using the R6 assay (see below). The volume of the test system was adjusted to 0.45 ml. The reaction mixture was then held at 37°C for 20 minutes before addition of 0.05 ml human serum. After 20 minutes, 2 ml cold saline were added and the samples were centrifuged to remove unlysed cells. The degree of lysis was determined spectrophotometrically at 541 mμ.

In some instances, C'5 activity was estimated by utilizing the ability of C'5 to reconstitute a sublytic dose of human serum. β_{1F}-globulin was reacted with 10^8 EAC'1a,4,2a cells in the presence of 0.05 ml human serum diluted 1:30 and 0.05 ml 0.1 M EDTA. The reaction volume was 0.5 ml. Lysis was allowed to proceed for 20 minutes at 37°C, and the degree of lysis was determined spectrophotometrically at 541 mμ.

Kinetic analysis of the formation of EAC'1a,4,2a,3,5,6: EAC'1a,4,2a,3 cells were reacted with C'5 and C'6, added simultaneously or in sequence of varying order. One ml of reaction mixture contained 2.2×10^8 cells, 20 μg β_{1C}, 40 μgβ_{1F}, C'6, and EDTA in a final concentration of 0.011 M. The temperature was 30°C. Samples of 0.45 ml containing 10^8 cells were withdrawn at timed intervals, transferred to 6 volumes of ice cold gelatin-veronal buffer, and centrifuged for 5 minutes at 4°C, whereupon the supernatants were discarded. The cells were resuspended in 0.45 ml gelatin-veronal buffer containing 0.011 M EDTA. Upon completion of sampling, all samples were incubated at 37°C for 20 minutes. Thereafter, each sample received 0.05 ml human serum and was incubated for an additional 10 minutes.

Assay of C'6 using complement-deficient rabbit serum: Serum from complement-deficient rabbits was shown to lack C'6 (14). Since it can be reconstituted fully with human C'6, such serum was used as a reagent for the assay of C'6. Two methods were used: (a) An aliquot of the test sample was allowed to react with 2.5×10^7 sensitized sheep cells and 0.15 ml of a 1:15 dilution of complement-deficient rabbit serum. The reaction volume was adjusted to 0.3 ml. Incubation at 32°C varied from 45 to 75 minutes in different experiments. Lysis was terminated by the addition of 1.5 ml cold saline followed by centrifugation. The degree of hemolysis was determined spectrophotometrically at 415 mμ. (*b*) EAC'1a,4,2a,3 cells were prepared by incubating 2×10^9 sensitized sheep erythrocytes with 0.2 ml complement-deficient rabbit serum in 10 ml of gelatin-veronal buffer. After 30 minutes' incubation at 32°C, 40 ml ice cold gelatin-veronal buffer was added. The cells were washed twice and resuspended in 8 ml gelatin-veronal buffer. 2.5×10^7 EAC'1a,4,2a,3 cells were set up with 0.15 ml complement-deficient rabbit serum diluted 1:15, test samples for C'6 determination and EDTA in a final concentration of 0.01 M. The reaction volume was 0.3 ml. After 45 to 75 minutes at 32°C 1.5 ml cold saline was added, the tubes were centrifuged and lysis was determined at 415 mμ.

Agglutination tests: A specific antiserum to β_{1F} was heat-inactivated and diluted in twofold steps from 1:5 to 1:320. One drop of diluted antiserum, 1 drop of a 1 per cent suspension of EAC'1a,4,2a,3,5,6 cells, and 1 drop of saline were mixed on a slide. After 10 minutes, agglutination was evaluated macroscopically.

RESULTS

Requirement of Several Serum Factors for the Conversion of EAC'1a,4,2a, to a Thermostable Intermediate Complex.—Preparations of β_{1C}-globulin, isolated according to a previously described method (2), have been shown to be effective in converting the thermolabile intermediate complex EAC'1a,4,2a to a thermostable state (3). Injection of such β_{1C} preparations into rabbits resulted in the production of antisera which contained antibodies to β_{1C}-globulin, but, also, to 2 other serum proteins. Fig. 1 shows an immunoelectrophoretic pattern of the 3 proteins in the euglobulin fraction of human serum. The 2 proteins which mi-

grate faster than β_{1C}-globulin are designated β_{1F}- and β_{1H}-globulin, respectively. Small amounts of these 2 proteins were consistently found to contaminate β_{1C} globulin prepared by the earlier method.

Correlating activity and protein distribution after electrophoresis of this ma-

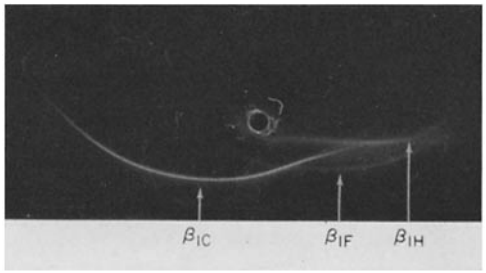

FIG. 1. Immunoelectrophoretic demonstration of antibodies to 2 additional serum proteins (β_{1F} and β_{1H}) in antiserum prepared to β_{1C}-globulin isolated according to the original method. Analysis was performed on human euglobulin; the anode was on the right.

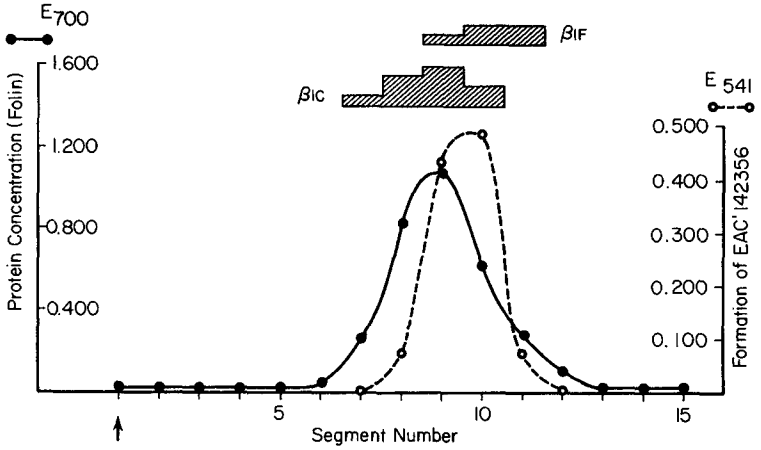

FIG. 2. Comparative electrophoretic analysis of EAC'1a,4,2a stabilizing activity, β_{1C}- and β_{1F}-globulin. The distribution of the activity correlates neither with β_{1C} nor with β_{1F}. Electrophoresis was performed on β_{1C}-globulin (prepared according to the original method) on a Pevikon block in phosphate buffer pH 7.0, T/2 = 0.1. Origin is indicated by arrow; the anode was at the right. Distribution of β_{1C} and β_{1F} was estimated immunologically.

terial on a Pevikon block at pH 7.0 showed the activity to be slightly faster than the protein peak (Fig. 2). Immunologic analysis of the block fractions indicated partial separation of β_{1F} and β_{1C} and revealed that the mobility of the activity was intermediate to that of the 2 proteins. This suggested that the activity is not resident in either protein, or that it results from the action of both components.

In the latter case, complete separation of β_{1C} and β_{1F} should be accompanied by loss of activity and recombination of the separated proteins should lead to its reconstitution. That this hypothesis was probably correct was suggested by the finding that combination of fractions 8 and 11 (Fig. 2), which, *per se*, contained little activity, gave rise to potentiation of their activity rather than to an additive effect.

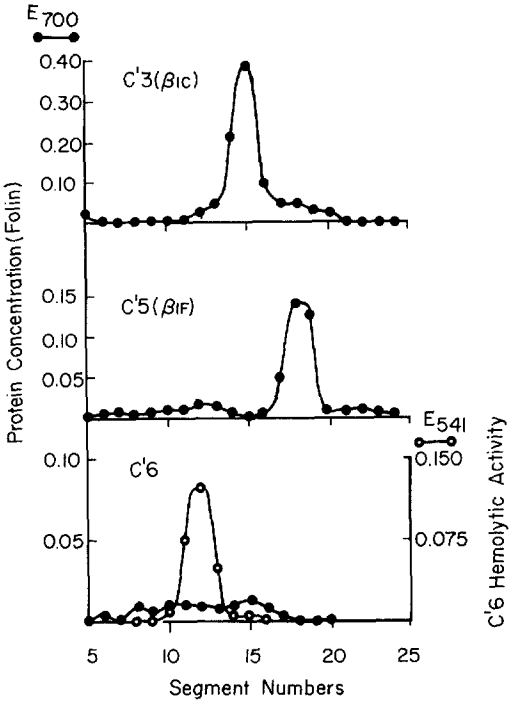

FIG. 3. Comparative electrophoretic analysis of purified β_{1C}- and β_{1F}-globulin and of partially purified C'6. Electrophoresis of the 3 samples was carried out simultaneously on the same Pevikon block for 33 hours in barbital buffer pH 8.6, T/2 = 0.05 and a potential gradient of 3.5 v/cm. Origin was at segment 1. β_{1C} and β_{1F} represented as protein (Folin), C'6 as activity.

After separation and purification of β_{1C}- and β_{1F}-globulin had been achieved with procedures to be described below, it became apparent that these 2 proteins were insufficient to convert EAC'1a,4,2a cells to a thermostable intermediate state. In the course of their purification, a third factor became separated from the 2 proteins which, as was found later, is also required for the conversion of EAC'1a,4,2a cells. This third factor is not identical with β_{1H}; at pH 8.6 it is electrophoretically slower than β_{1C}, although it overlaps to some extent with it. A precipitating antibody to this component, which is called C'6, has not

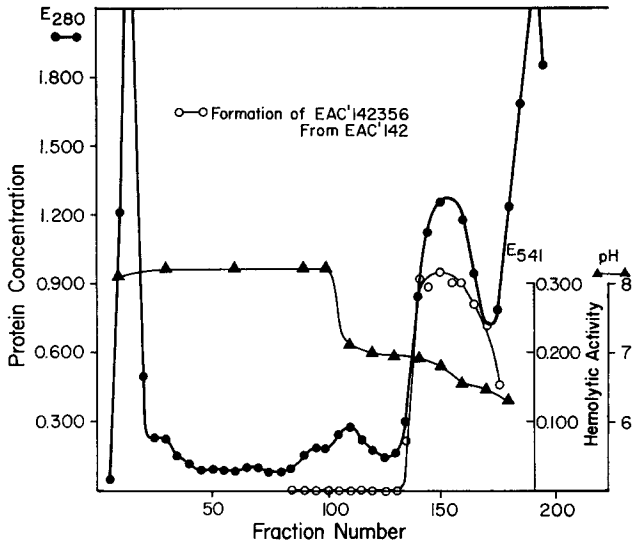

FIG. 4. Detection of EAC'1a,4,2a stabilizing activity (C'3, C'5, and C'6) in euglobulin fractions after chromatography on TEAE-cellulose. Gradient was started at tube 80. By immunologic analysis, β_{1C} and β_{1F} were detected in fractions 140 through 180. Fractions 140 through 160 were pooled and concentrated for further separation on hydroxyl apatite.

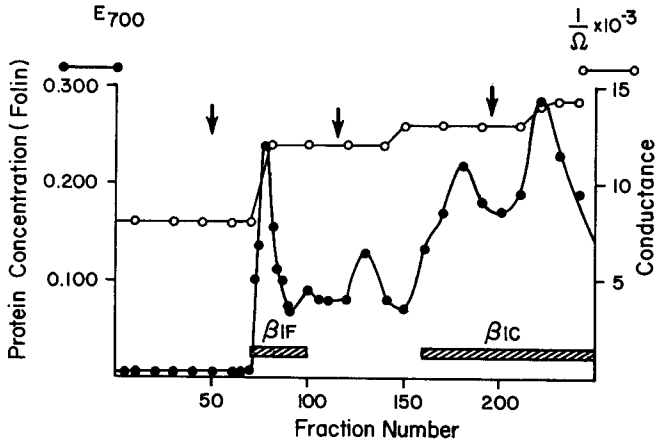

FIG. 5. Chromatographic separation of β_{1C}- and β_{1F}-globulin on hydroxyl apatite. The protein (fractions 140 to 160, Fig. 4) was applied in PO_4 buffer adjusted to a conductance of 8 mmhos/cm. It was eluted stepwise by buffers of increasing PO_4 concentration. The arrows indicate change of buffer, and the bars the distribution of β_{1C} and β_{1F} as estimated immunologically. Fractions 70 to 90 were pooled and concentrated for further separation by zone electrophoresis.

been found to date in spite of several attempts to produce anti-C'6 antibodies in rabbits by injecting partially purified preparations. Identification of C'6 with an individual serum protein has not yet been achieved. However, work on its final purification and characterization is in progress.

Fig. 3 demonstrates the electrophoretic patterns of the 3 components required for the conversion of EAC'1a,4,2a to a thermostable state hereafter described as EAC'1a,4,2a,3,5,6. β_{1C} or C'3 and β_{1F} or C'5 are represented as proteins, C'6 as activity. The necessity of C'3, C'5, and C'6 for the conversion of EAC'1a,4,2a to a thermostable complex follows from the observation that omission of any one of the 3 factors from the reaction mixture results in failure to produce a thermostable complex (see below).

TABLE I

Purification of β_{1F}

1. Preparation of euglobulin	Precipitated from fresh human serum dialysed against PO_4 buffer, pH 5.4, T/2 = 0.02.
2. TEAE chromatography	PO_4 buffer gradient elution (0.03 M, pH 8.1 → 0.25 M NaH_2PO_4).
3. OH apatite chromatography	PO_4 buffer, pH 7.9, stepwise elution (conductance: 8, 12, 13, and 14.5 m-mhos).
4. Preparative electrophoresis	Pevikon block, barbital buffer, pH 8.6, T/2 = 0.05.

Isolation of B_{1F}*-Globulin.*—Since β_{1F} is a euglobulin, like β_{1C}, this fraction of serum was used as starting material. Chromatography of the euglobulin fraction on TEAE-cellulose allowed the separation of β_{1F} from most proteins present except β_{1C} and β_{1H}. The 3 proteins were eluted together in a pH range of 6.8 to 6.0. Although C'6 activity was, in part, eluted with earlier fractions, considerable amounts of this component overlapped with β_{1F} and β_{1C}, thus giving rise to complete EAC'1a,4,2a stabilizing activity in fractions 140 to 180 of the chromatogram shown in Fig. 4.

To separate all 3 proteins from each other and to obtain C'6-free β_{1F} and β_{1C} chromatography on hydroxyl apatite was used. A stepwise elution procedure was worked out employing increasing salt concentration at pH 7.9 (Fig. 5). According to this method, β_{1F} is eluted at a conductance of 12 mmhos/cm, β_{1H} and C'6 at a conductance of 13 mmhos/cm. The bulk of the β_{1C} is eluted in a final step at a conductance of 14.5 mmhos/cm. There is trailing of β_{1H}-globulin but not of C'6 into the β_{1C} eluates so that only the second half of this step contains pure β_{1C}-globulin.

Concentrated fractions of β_{1F} obtained by OH apatite chromatography con-

tain less than 10 per cent contaminants, as estimated by preparative electrophoresis and sucrose density grandient ultracentrifugation. Traces of γ_G- and α_2-globulins can be shown by immunoelectrophoresis. In order to eliminate

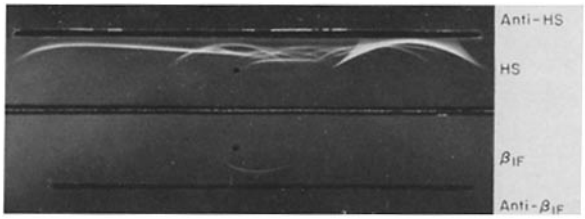

FIG. 6. Immunoelectrophoresis of highly purified β_{1F}-globulin. For comparison, the pattern of whole human serum is also shown. Top trough: rabbit anti-whole human serum. Middle trough: empty. Bottom trough: rabbit anti-β_{1F}-globulin. Anode was on the right.

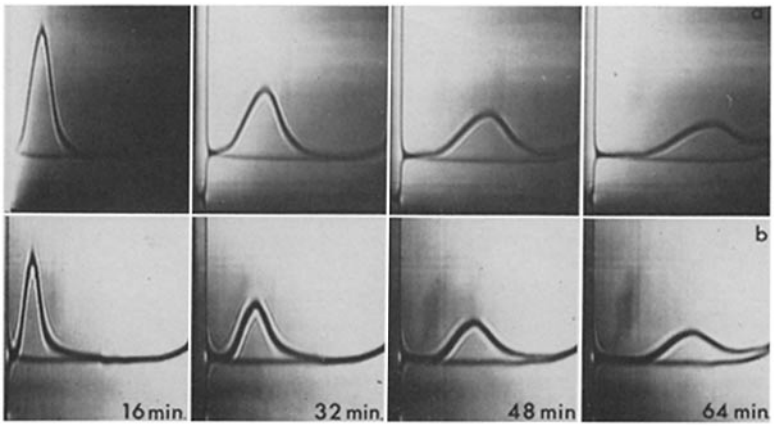

FIG. 7. Schlieren patterns of isolated β_{1F}-globulin photographed at different times during ultracentrifugation at 52,640 RPM: (*a*) 11 mg/ml, and (*b*) 6 mg/ml. Note tendency to spreading and asymmetry of boundaries.

these last impurities, the hydroxyl apatite purified β_{1F} was subjected to preparative electrophoresis using Pevikon as supporting medium and barbital buffer, pH 8.6, T/2 = 0.05. After 35 hours at 3.5 v/cm, β_{1F}-globulin could be eluted from the block in highly purified form. Table I summarizes the entire procedure of isolation.

Partially purified C'6 was obtained by TEAE chromatography of the euglobulins. It was eluted from the column between pH 7.4 and 6.8 preceding elution of β_{1C}, β_{1F}, and β_{1H}. Immunologic analysis of partially purified C'6 revealed the presence of γG- and of γA-globulin but of no other major serum protein that can be detected with an antiserum to whole human serum.

Some Properties of β_{1F}-Globulin.—Purified β_{1F}-globulin was tested by Ouchter-

lony's double diffusion technique against a variety of antisera. A single precipitin line was formed with an antiserum to whole human serum and with specific anti-β_{1F} serum. The 2 lines fused completely with each other, showing a reaction of identity. Negative reactions were obtained with antisera prepared to γA-, γM-, γG-globulin, transferrin, β_{1C}-, β_{1E}-globulin, fibrinogen, and β-lipoprotein. The preparations were homogeneous also by immunoelectrophoretic criteria. Fig. 6 demonstrates the position of the β_{1F} precipitin line in relation to the pattern of whole human serum. Using an anti-whole human serum, β_{1F} could not be detected in human serum. This is due to the low serum concentration of β_{1F} as

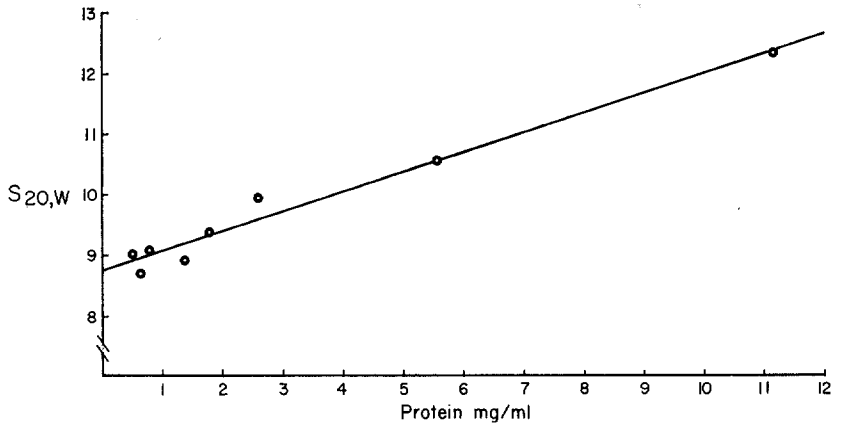

FIG. 8. s *versus* c plot for β_{1F}-globulin, showing an anomalous concentration dependence of the s-rate. By least-square analysis, y = 0.33 x +8.7.

well as to insufficient amounts of β_{1F} antibody in antiserum against whole human serum. Analysis of aged β_{1F} preparations (stored at + 4°C for 1 month or more) revealed immunologic and electrophoretic heterogeneity. Part of the protein was converted to a faster migrating component which showed partial immunologic identity with native β_{1F}-globulin. The nature of this product awaits further studies.

Ultracentrifugal analysis of 3 different preparations at various concentrations disclosed a single boundary in each instance, precluding the presence of a readily detectable contaminant. There was, however, a marked tendency to boundary spreading and to boundary asymmetry, as can be seen in Fig. 7. Comparing the schlieren patterns of the same preparation at 2 different protein concentrations, it is apparent that at higher concentration the peak of the boundary is shifted toward the bottom and at lower concentration toward the top of the cell. This phenomenon indicates a tendency of the β_{1F} molecules to interact with each other and to form polymers at higher protein concentration. The anomalous concentration dependence of the s-rate is depicted in Fig. 8. The slope of the s

vs. c plot is +0.33 and the sedimentation coefficient at infinite dilution, $s^0_{20,w}$ = 8.7 S.

Comparison of Physicochemical Properties of β_{1F}-Globulin and of C'5.— Throughout the procedure of isolation a close correlation was found to exist between the distribution of β_{1F}-globulin and of C'5 activity. Although this sup-

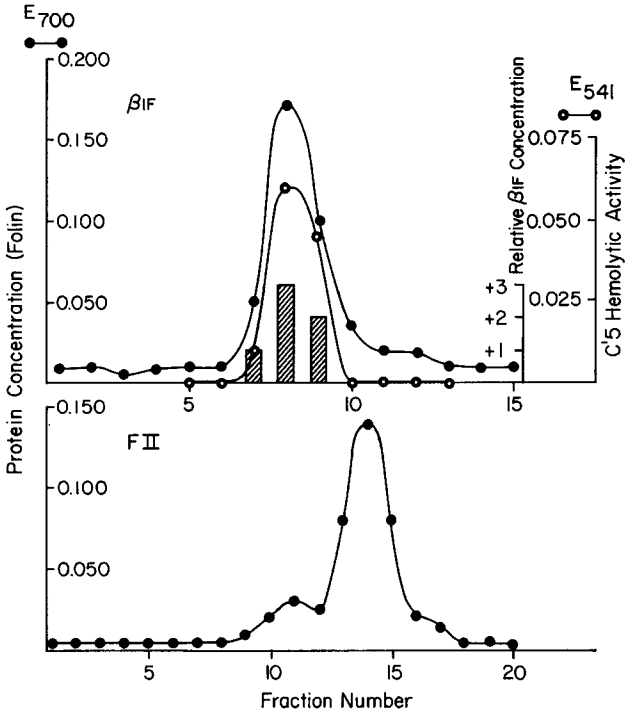

FIG. 9. Correlation between the distribution of C'5 hemolytic activity and β_{1F}-globulin upon ultracentrifugation in a sucrose density gradient. Note correspondence between major protein peak, immunologically estimated β_{1F}-globulin and C'5 activity. For comparison, the pattern of γG-globulin (Cohn fraction II) is shown. Sedimentation is toward the left.

ported the hypothesis of their identity, the possibility could not be ruled out entirely that the activity was due to a contaminant present in β_{1F} preparations. In order to test whether C'5 activity could be dissociated from β_{1F}, separation techniques were employed which differed from those used in the isolation procedure. Fig. 9 demonstrates the results obtained when a β_{1F} preparation obtained by OH apatite chromatography was subjected to zone ultracentrifugation. The position of the β_{1F} peak is identical to that of C'5 activity.

Starch gel electrophoresis also failed to separate C'5 and β_{1F} (Fig. 10). The material used in this experiment was prepared by TEAE chromatography and

electrophoresis omitting OH apatite chromatography. It contained a small amount of β_{1A}, which is visible on the stained gel as a band in front of β_{1F}. Thus, all separation procedures employed failed to dissociate C'5 activity and β_{1F}-globulin.

Characterization of C'5 Activity.—Requirement of C'5 for the conversion of EAC'1a,4,2a cells to a thermostable state is demonstrated in Table II. This table lists the design and the results of experiments performed to determine the position of C'5 in the complement reaction sequence. EAC'1a,4,2a cells were

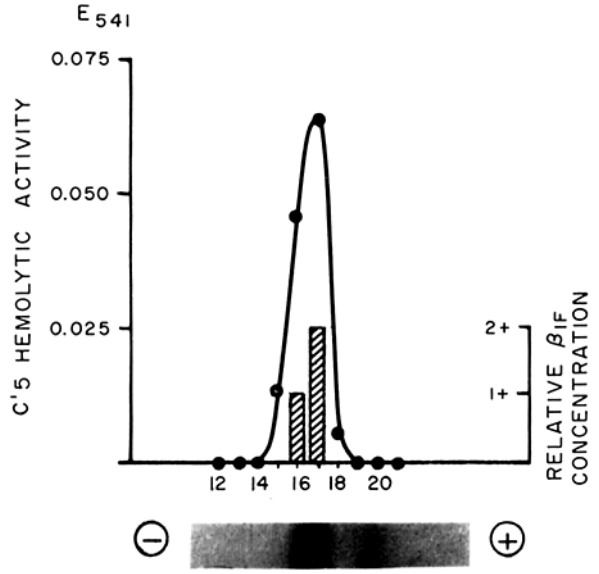

FIG. 10. Correlation between the distribution of C'5 hemolytic activity and β_{1F}-globulin upon starch gel electrophoresis. C'5 activity and immunologically demonstrable β_{1F}-globulin occupy the same position in the gel as the major protein band. The faster migrating band was caused by contaminating β_{1A}-globulin (see text). Origin at segment 1. After electrophoresis, the gel was sliced into 2 halves, one was stained for protein, the other was cut into suitable segments which were eluted and analyzed for C'5 and β_{1F}.

first treated with 1 or a combination of 2 of the 3 stabilizing factors, C'3, C'5, and C'6. Thereafter, the cells were washed, resuspended, and held at 37°C for a sufficient period of time to allow decay of non-stabilized cells. During this second period of incubation, the cells were exposed to those factors which were not present in the reaction mixture during the first period of treatment. The proportion of thermostable intermediate complexes was then determined by the degree of lysis ensuing upon addition of EDTA serum. Two conclusions can be drawn from these results. A thermostable intermediate complex is formed only if C'3, C'5, and C'6 are added together to EAC'1a,4,2a cells, or if C'5 and C'6

are reacted with EAC'1a,4,2a,3 cells. Secondly, regarding the sequence of action of the 3 complement factors, it is apparent that C'3 precedes C'5 and C'6 and that C'3 is the only one capable of reacting directly with EAC'1a,4,2a cells.

C'5 and C'6 appear to be functionally dependent upon each other, since an intermediate complex containing C'5, but not C'6, could not be prepared. However, kinetic analysis of the interaction of C'5 and C'6 with EAC'1a,4,2a,3 cells (Fig. 11) indicated that previous incubation of C'5 with the cell complex facilitated the subsequent reaction with C'6. Since reversal of the sequence of

TABLE II

Determination of the Sequence of Action of C'3, C'5, C'6 on EAC'1a, 4, 2a Cells

First treatment	Second treatment*	Lysis with EDTA serum
5 min. at 30°C in presence of	20 min. at 37°C in presence of	
C'3 + C'5	C'6	No
C'3 + C'6	C'5	No
C'5 + C'6	C'3	No
C'3	C'5 + C'6	Yes
C'5	C'3 + C'6	No
C'6	C'3 + C'5	No
C'3 + C'5 + C'6		Yes
	C'3 + C'5 + C'6	Yes

* Cells were washed between first and second treatment.

addition resulted in a lag of onset of EAC'1a,2a,3,5,6 formation, β_{1F}-globulin was considered to precede functionally C'6 and was therefore designated C'5. It will be seen below that this definition of sequence of action may be somewhat artificial in view of the tendency of the 2 components to occur in the form of a complex in free solution.

C'5 activity is further characterized by a pronounced thermolability. Incubation of purified C'5 for 10 minutes at 56°C resulted in 90 per cent inactivation. A similar thermolability was not observed with C'6.

Quantitation of C'5 activity was performed in 2 systems; one measuring stabilization of EAC'1a,4,2a cells at 37°C in the presence of C'3 and partially purified C'6; the other determining lysis of EAC'1a,4,2a cells in the presence of an amount of EDTA serum, *per se* insufficient to cause lysis. The 2 dose-response curves obtained are shown in Fig. 12. In both systems, relatively large amounts of purified β_{1F}-globulin (C'5) were required to achieve a significant degree of lysis, which is in contrast to the effectiveness of less purified preparations. The

possibility exists, therefore, that part of the protein became inactivated in the course of the long purification procedure or that a cofactor was removed in the process, which exerts an enhancing effect on C'3, C'5, or C'6. The possibility of a missing cofactor is suggested by the observation that glycine in a concentration of 0.05 M increases the stabilizing activity of C'3, C'5, and C'6 by 60 per cent. Addition of the ultrafiltrate of fresh human serum or the supernatant of boiled serum did not result in a similar enhancement of EAC'1a,4,2a stabilization.

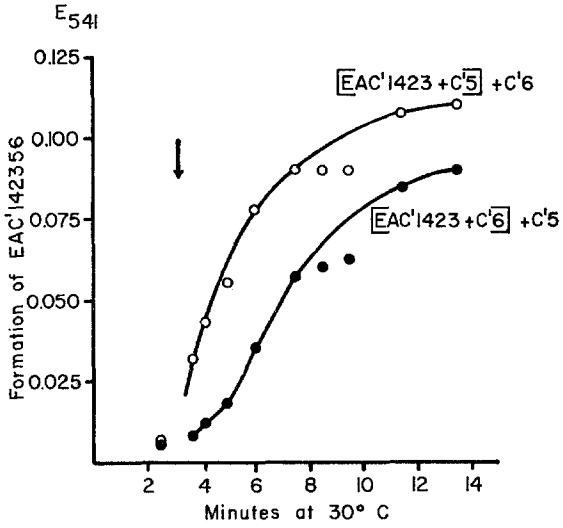

FIG. 11. Kinetic analysis of the formation of the EAC'1a,4,2a,3,5,6 complex from EAC'1a,4,2a,3 cells and C'5 and C'6. The order of addition of the 2 components was varied to determine their sequence of action. The open circles represent values obtained on a reaction mixture which initially contained EAC'1a,4,2a,3 cells and C'5. After 3 minutes, C'6 was added and immediately thereafter sampling was begun. The solid circles represent values obtained on EAC'1a,4,2a,3 initially incubated with C'6, whereupon C'5 was added to the reaction mixture. The arrow indicates the time of addition of C'5 and C'6, respectively. Each sample withdrawn contained 10^8 cells, which had reacted with 15 μg β_{1F} and an active dose of C'6. 100 per cent lysis corresponds to OD 541 = 0.260.

While the effect of C'3, C'5, and C'6 on EAC'1a,4,2a has been described as stabilization, it is not known which moiety of this cell-complement complex is actually affected. Since EAC'1a,4,2a cells tend to revert to the EAC'1a,4 state, it was conceivable that C'3, C'5, and C'6 would act by preventing dissociation of C'2a. In this case, C'2a should remain associated with the stabilized complex (EAC'1a,4,2a,3,5,6). Alternatively, C'3, C'5, and C'6 convert EAC'1a,4,2a cells to a state in which interaction with subsequent complement components does no longer depend upon the presence of C'2a. To distinguish between the 2 possibilities, various intermediate complexes were tested for their ability to

convert β_{1C}-globulin to β_{1G}-globulin. This reaction has previously been shown to be catalyzed by cell bound C'2a (15). It was found that cells that have reacted with C'3, C'5, and C'6 and that are stable with respect to the effect of subsequent components, are unable to convert β_{1C}-globulin. This experiment shows that EAC'1a,4,2a cells, after their stabilization by C'3, C'5, and C'6, may be devoid of C'2a activity. Thus, C'3, C'5, and C'6 do not seem to act by stabilizing C'2a activity.

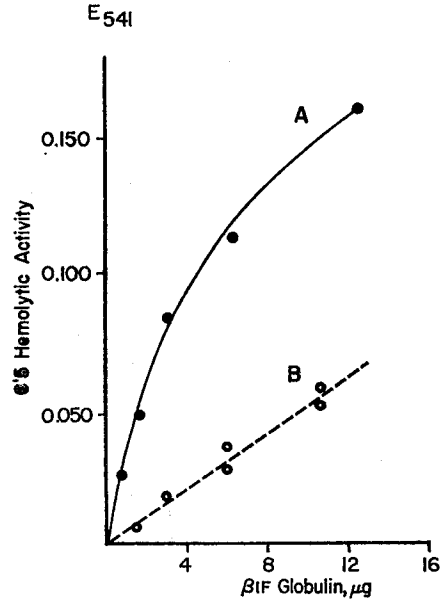

FIG. 12. Dose response curves obtained with purified β_{1F}-globulin. C'5 hemolytic activity was measured by two different hemolytic assays: (*A*) the dilute serum system; (*B*) the EAC' 1a,4,2a stabilizing system (in the presence of appropriate amounts of C'3 and C'6). Lysis of 10^8 cells correspond to $OD_{541} = 0.260$. 65 per cent of the cells used for these assays were in the EAC'1a,4,2a state.

Physicochemical Interaction between β_{1F}-Globulin (C'5) and C'6.—Considering the s-rates of C'3, C'5, and C'6, which are 9.5, 8.7, and 5S, respectively, EAC'-1a,4,2a stabilizing activity should be found after density gradient ultracentrifugation somewhere in the 9.5 to 5S region, provided the 3 factors do overlap. Contrary to this assumption, stabilizing activity was located to a zone sedimenting slightly faster than C'3. This finding suggested the occurrence of protein-protein interaction between at least 2 of the 3 factors concerned, resulting in the formation of a faster sedimenting complex. Subsequent analysis revealed a tendency of C'6 to complex with C'5, but not with C'3. As demonstrated in Fig. 13, partially purified C'6 sedimented as a single component in the absence

of C'5. However, upon addition of C'5, C'6 activity distributed bimodally, with one activity peak corresponding to the original material and the other sedimenting more rapidly. Since the fast C'6 component sedimented together with C'5, it is concluded that it is present in the form of a complex with C'5, and that

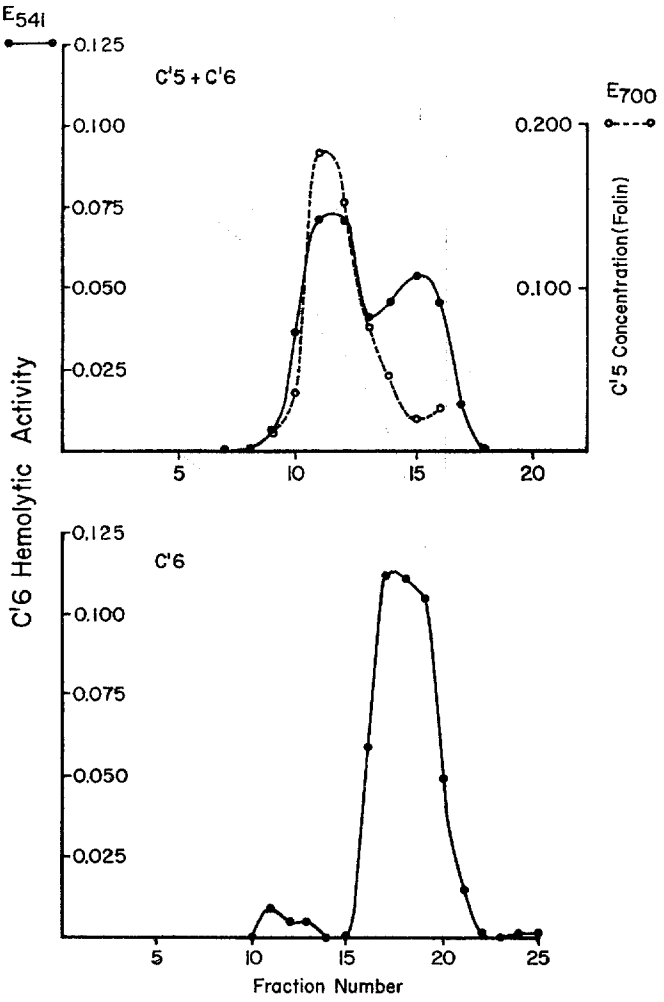

FIG. 13. Influence of β_{1F}-globulin (C'5) on the sedimentation behavior of C'6 upon zone ultracentrifugation. Bottom pattern represents partially purified C'6, without β_{1F}, and the top pattern an identical amount of the same preparation of C'6 to which purified β_{1F} was added. Ultracentrifugation was carried out at 35,000 RPM for 18 hours in a 10 to 40 per cent sucrose density gradient prepared in phosphate buffer pH 7.0, T/2 = 0.1. β_{1F}-Globulin (C'5) of top graph is represented by protein. Direction of sedimentation is toward the left.

slow C'6 represents free material. In experiments where the amount of C'6 was diminished or the amount of C'5 increased only the fast sedimenting C'6 activity peak was observed. The complex persisted in the presence of 0.01 M EDTA. Detection of C'6 activity was facilitated in these experiments by the use of complement-deficient rabbit serum which was found to lack C'6 but none of the other complement components (14).

To preclude the possibility that the observed interaction resulted from partial denaturation of either of the 2 components, fresh human serum was examined for the occurrence of a similar complex. In 2 different sera tested, the major portion of C'6 activity sedimented with an s-rate similar to that of the C'5-C'6 complex.

TABLE III

Uptake of I^{131}-Labeled β_{1F}-Globulin (C'5) by Various Intermediate Complexes

Experiment	Intermediate complex (1.5×10^8 cells)	Complement factors present	Amount of β_{1F} I^{131}	Uptake of β_{1F} I^{131}	
			CPM	CPM	*per cent*
IA*	EAC'1a,4,2a	C'3, C'5, C'6	228,000	672	0.3
B	EAC'1a,4,2a	C'3, C'5 (56°C), C'6	228,000	2316	1.0
IIA‡	EAC'1a,4,2a,3	C'5, C'6	17,850	820	4.6
B	EAC'1a,4	C'5, C'6	17,850	760	4.3

* EAC'1a,4,2a cells were reacted for 30 minutes at 37°C (A) with hemolytically active β_{1F}-globulin and (B) with heat-inactivated β_{1F}-globulin (56°C for 10 minutes). In A, all available EAC'1a,4,2a cells (41 per cent of the total cell population) were converted to EAC'1a,4,2a,3,5,6, in B less than 5 per cent. Specific radioactivity was 4700 CPM/μg β_{1F}.

‡ Specific radioactivity was 1490 CPM/μg β_{1F}.

Immunoelectrophoretic Analysis of β_{1F} Following Various Treatments of Serum.—Immunoelectrophoresis disclosed a complete disappearance of the β_{1F} precipitin line after incubation of serum for 1 hour at 56°C. Treatment with zymosan (2.5 mg/ml), or with a bovine albumin-rabbit anti-bovine albumin precipitate for 2 hours at 37°C, led to diminution, but not to disappearance of the line. Dilute hydrazine (0.016 M) which has previously been shown to alter the immunoelectrophoretic pattern of β_{1E}- and of β_{1C}-globulin (11, 16) had no apparent effect on β_{1F}.

Analysis of Complement Treated Immune Complexes for β_{1F}-Globulin.—Since certain complement components were shown previously to enter into physical union with immune complexes, the question arose as to whether β_{1F}-globulin exhibits a similar behavior. Uptake of β_{1F} by antigen-antibody complexes was, indeed, suggested by the diminution of the β_{1F} precipitin line, as detected by immunoelectrophoresis following treatment of serum with antigen-antibody complexes or zymosan. To test this hypothesis cells in the stage EAC'1a,4,2a,

3,5,6 were carefully washed and reacted with a series of dilutions of a specific antiserum to β_{1F}. No agglutination was detectable. Further, in no experiment where EAC'1a,4,2a,3,5,6 cells were prepared with I^{131}-labeled β_{1F} could any uptake significantly higher than that of the controls be demonstrated (Table III). Similar results were obtained when an antigen-antibody precipitate (bovine serum albumin–rabbit anti-bovine serum albumin) was tested which had been treated with human serum. After careful washing the precipitate was dissolved by addition of an excess of the antigen, and analyzed in an Ouchterlony plate prepared from agar containing 35 mg bovine serum albumin per ml. Although an antiserum specific for β_{1C} demonstrated uptake of this complement component, no precipitin line was formed with the specific anti-β_{1F}-antiserum.

DISCUSSION

The complexity of the classical third component of hemolytic complement has been known for some time (17, 18). Linscott and Nishioka (19) demonstrated 4 subcomponents, whereas Wellensiek and Klein (20) found 5 and Nelson and coworkers (21) 6 factors. The classical third component acts, according to Mayer (13), on the EAC'1a,4,2a complex, which, in the course of this reaction, is converted to E*. In this state the cell undergoes spontaneous lysis. One of the characteristics of the EAC'1a,4,2a cell is its lability at 37°C (13). As Mayer (22, 23) has shown, C'2 dissociates from the complex and the cells revert to the EAC'1a,4 state. Somewhere midway during the conversion of EAC'1a,4,2a cells to E*, the cells assume thermostability. In this intermediate state they can be held at 37°C for prolonged periods without undergoing spontaneous lysis and without losing susceptibility to the lytic action of subsequent complement factors. The thermostable intermediate complex is a convenient tool for the investigator, as it permits division of the reaction of the classical third component into two major phases. The first phase leads to the formation of a stable complex from EAC'1a,4,2a cells the second phase to the conversion of the stable complex to E*. The present study is concerned only with the first phase of the reaction.

Originally it was thought that a single protein, β_{1C}-globulin, was responsible for conversion of EAC'1a,4,2a cells to a stable complex (3). Later, 2 components were recognized as essential in this reaction, in human serum β_{1C}- and β_{1F}-globulin (1), and in guinea pig serum C'3c and C'3b (20). In this paper a third factor was pointed out to participate in the stabilization reaction. With reference to their activity, these 3 components of human serum have been designated C'3, C'5, and C'6 (4, 23, 24). It is emphasized that C'3 denotes the activity of β_{1C}-globulin and no longer that of the classical third component of complement.

The chief purpose of this paper was to report a method of isolation of β_{1F}-globulin and to present evidence indicating that this protein is a component of

the hemolytic complement system. The complement component activity of β_{1F}-globulin is primarily manifested in the requirement of this protein for the conversion of EAC'1a,4,2a cells to the stable complex EAC'1a,4,2a,3,5,6. In a large number of experiments it became evident that a stable intermediate complex could not be prepared from EAC'1a,4,2a cells unless β_{1F}-globulin was present in the reaction mixture. The relatively low efficiency of β_{1F}-globulin in the stabilizing system, however, suggests that a cofactor might be missing or that the protein was partially inactivated during isolation. This question is presently under investigation.

Other observations, not reported in this paper, also support the view that β_{1F}-globulin is a component of complement. The serum of certain strains of mice have been found to lack hemolytic complement activity (25) as well as one of the β-globulins (26, 27). The defect was shown to be under genetic control (28). The deficient murine serum protein designated MuB1 (27) was postulated to be a complement component. Its absence in complement deficient mice is considered to be the cause for the apparent complement deficiency. Since Terry *et al.* (29) found the first, second and fourth component intact, the defect could only involve one of the later reacting complement components. Using mouse anti-MuB1 and mouse anti-human β_{1F}, it was possible to demonstrate a specific immunologic relation between MuB1 and human β_{1F} (30). Thus, MuB1 appears to be the murine analogue to β_{1F}, the fifth component of human complement.

Regarding the mode of action of β_{1F}-globulin, the following tentative conclusions can be drawn. β_{1F} participates in immune hemolysis without being physically incorporated into the cell-complement complex. This behavior suggests that β_{1F} plays a mediating role in the complement reaction which is similar to that of an enzyme. As the kinetic data indicated, β_{1F} is capable of interacting with EAC'1a,4,2a,3 cells. However, the cells do not seem to be affected by this interaction, since an EAC'1a,4,2a,3,5 complex could not be isolated from the reaction mixture. Instead, it appears that EAC'1a,4,2a,3 cells act on β_{1F} conditioning this protein for its subsequent effect on the cells, which, however, ensues only after addition of C'6.

In view of the dependence of the β_{1F} effect upon C'6, it was thought probable that both factors form a complex in the fluid phase before interacting with cells. A β_{1F}-C'6 complex could indeed be demonstrated in solutions containing both components in purified form. Evidence was also obtained for the occurrence of a similar complex in fresh human serum. This finding, in addition to the kinetic data and the failure to prepare EAC'1a,4,2a,3,5 cells, suggest that formation of the β_{1F}-C'6 complex is a prerequisite for the function of the fifth and the sixth component of complement.

The result of the interaction of C'5,6 with EAC'1a,4,2a,3 cells is the stable complex symbolized by the expression EAC'1a,4,2a,3,5,6. The term "stable complex" is actually a misnomer. It refers to the lability of the C'2a moiety of

EAC'1a,4,2a cells and implies stabilization of this component. Stabilization of C'2a is apparently not effected by C'5,6. If it were, EAC'1a,4,2a,3,5,6 cells should retain the ability to convert β_{1C}-globulin. This, however, was not observed. It is therefore tempting to speculate that C'5,6 instead of stabilizing EAC'1a,4,2a,3 exert a direct effect on the surface of these cells rendering it irreversibly susceptible to the action of the terminal components.

SUMMARY

At least 3 complement factors were found necessary for the conversion of the thermolabile intermediate complex EAC'1a,4,2a to a thermostable state. One of these factors is the earlier described β_{1C}-globulin. The second, a heretofore unrecorded serum protein, β_{1F}-globulin. The third factor has not yet been defined as a discrete serum protein entity. Kinetic experiments indicated that β_{1C} reacted prior to β_{1F}, which in turn seemed to precede the third factor in the reaction sequence. Therefore, the 3 components were tentatively designated the third (C'3), the fifth (C'5), and the sixth (C'6) components of complement, respectively. A procedure was developed allowing the isolation of highly purified β_{1C}-(C'3) and β_{1F}-globulin (C'5) and of partially purified C'6.

With respect to its function in immune hemolysis, β_{1F}-globulin or C'5 was found to be closely dependent on the simultaneous presence of C'6. The hypothesis that C'5 and C'6 form a functional unit was supported by the finding that both components interact with each other in solution resulting in the formation of a complex. A similar complex was also found in fresh human serum.

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