ISOLATION AND DESCRIPTION OF THE FOURTH COMPONENT OF HUMAN COMPLEMENT*

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To elucidate the chemical mode of action of complement and the nature of its damaging effect on cell membranes, it is essential to obtain information on the molecular properties of individual complement components. Since Heidelberger (1) demonstrated that antigen-antibody complexes take up nitrogen from fresh serum, it is generally held that the chemical correlative of complement activity is protein. The identity of the proteins with complement activity, however, has largely remained obscure due to the great number of different protein species occurring in serum. The purpose of the present study was to elaborate a method for the isolation of one of these, the fourth component of human complement (C′4) and, if possible, to identify this activity with a physically and immunologically homogeneous serum protein entity.

The existence of the fourth component of complement was postulated in 1926 by Gordon, Whitehead, and Wormall (2). A lipase preparation which was suspended in buffer containing ammonia was found to abolish hemolytic activity of serum without affecting the activities of any of the three components of complement known at that time. At first this finding was interpreted to indicate that an unknown complement factor had been destroyed by the action of lipase. Subsequently it was demonstrated, however, that the new factor was sensitive to ammonia and not to lipase. Thus the classical fourth component was discovered and characterized on the basis of its sensitivity to ammonia and other primary amines. C′4 is further characterized by its position in the sequence of the complement reaction steps. It is preceded by the first component (C′1) (3–5) and followed by the second (C′2) (6). The chemical mode of action of C′4 is as obscure as that of the other complement components, excepting one moiety of C′1 which exhibits esterase (7, 8) and peptidase (9) activity.

In the following the isolation of a heretofore unrecorded serum protein will be reported, which on the basis of its immunoelectrophoretic behavior was designated β_{1E}-globulin. Some of its properties will be described and evidence

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will be presented indicating that β_{1E}-globulin represents the fourth component of human hemolytic complement.

Materials and Methods

Serum and Serum Fractions.—Serum prepared sterilely from the blood of white donors was purchased from Knickerbocker Blood Bank, Inc., New York. Processing was begun on the day the blood was taken.

Pseudo- and euglobulin fractions of serum were prepared by dialyzing 500 ml of serum for 15 hours at 4°C against 2 × 20 liters of phosphate buffer, pH 5.4, $\frac{\tau}{2} = 0.02$. The euglobulin fraction was removed by centrifugation in an International refrigerated centrifuge at 1560 g and 4°C for 30 minutes. It was washed twice with the above buffer and served thereafter as starting material for the preparation of β_{1C}-globulin. The supernatant pseudoglobulin fraction was used as starting material for the isolation of β_{1E}-globulin.

β_{1C}-Globulin.—This protein was isolated from the euglobulin fraction of fresh serum according to methods described previously (10, 11).

Rabbit Antiserum to β_{1E}-Globulin.—Rabbits were twice injected subcutaneously with 1 to 2 mg of highly purified β_{1E}-globulin in complete Freunds' adjuvant at an interval of 2 months. They were bled 1 month after the second injection. The sera contained between 0.5 and 4 mg of antibody protein per ml and gave a single precipitin line with whole human serum upon immunoelectrophoresis and Ouchterlony's double diffusion test.

Reagent for the Detection of the Fourth Component of Complement.—To inactivate the fourth component of complement fresh human serum was treated with hydrazine at a final concentration of 0.01 M at 37°C for 90 minutes. This procedure has previously been shown to inactivate also β_{1C}-globulin, the most hydrazine-sensitive moiety of the third component of human complement (12). To restore the normal concentration of β_{1C}-globulin in hydrazine-treated serum, approximately 250 μg of purified protein was added to 1 ml R4 immediately before use. Alternatively, R4 and β_{1C} were added separately to the reaction mixture.

Chromatography.—500 ml of pseudoglobulin dialyzed over night against 4 liters of starting buffer were applied to a 8 × 50 cm column containing 1500 ml of packed TEAE cellulose, equilibrated with 0.02 M phosphate buffer, pH 7.3 (starting buffer). After adsorption of the applied material by the cellulose, the column was washed with 4 liters of starting buffer, containing 0.11 M NaCl until the conductivity of the effluents had reached 12,000 micromhos/cm. The effluent was discarded. The protein which under these conditions remains adsorbed to cellulose and which included β_{1E}-globulin, was then eluted by a salt gradient elution procedure. The mixing chamber, a 2000 ml beaker, contained 2000 ml of 0.11 M sodium chloride in starting buffer. It was connected by syphon with a 2000 ml Erlenmeyer flask containing 2000 ml of 1.5 M sodium chloride in starting buffer. Fractions of 280 drops were collected and the flow rate was 100 to 120 drops per minute.

After electrophoretic purification of C'4 activity containing column fractions the material was rechromatographed on a 1.1 × 48 cm column filled with 45 ml of packed TEAE cellulose, equilibrated with starting buffer. Salt gradient elution was started immediately after application of the protein. As mixing chamber was used a 250 ml beaker containing 180 ml starting buffer which was connected by syphon to a 125 ml Erlenmeyer flask containing 120 ml 0.7 M NaCl in starting buffer. Fractions of 30 drops were collected at a flow rate of approximately 10 drops per minute. Both of the chromatographic procedures were performed strictly at 4°C.

Preparative Electrophoresis.—Fractions of the initial chromatographic procedure containing C'4 activity were pooled and concentrated by ultrafiltration to approximately 10 ml. This material was then applied to a 1 × 20 × 50 cm block of Pevikon C-860 in barbital buffer,

pH 8.6, $\frac{\Gamma}{2}$ = 0.05 (13). Electrophoresis was carried out at 4°C for 20 hours, employing 3.5 V/cm.

Immuno- and Starch Gel Electrophoresis.—Immunoelectrophoresis was carried out according to Scheidegger (14). In addition to anti–β_{1E}-globulin sera, two anti–whole human sera were used, one produced by the Behringwerke A.G., Marburg. The other by the Institute Pasteur, Paris.[1] Starch gel electrophoresis was performed as described by Smithies (15) using the discontinuous buffer system introduced by Poulik (16). Approximately 100 to 200 μg of purified protein was applied by means of a filter paper carrier. Electrophoresis was carried out at 4°C for 3 hours in an electric field of 11 V/cm.

In a few instances, protein was eluted from small segments of the gel by freezing and thawing and subsequent centrifugation. The yield was less than 5 per cent of the material applied which, however, was sufficient for C′4 activity determinations.

Ultracentrifugation.—Sedimentation coefficients were determined in a Spinco model E analytical ultracentrifuge. Density gradient ultracentrifugation was carried out as described by Kunkel (17).

Estimation of the Fourth Component of Complement.—To localize C′4 activity in fractions obtained by chromatography, electrophoresis, and zone ultracentrifugation, suitable aliquots were incubated with 2.5 to 2.75 $\times 10^8$ EAC′1 cells (complex of sensitized sheep cells and the first component of complement), in a total volume of 0.5 to 0.6 ml at 37°C for 20 minutes. The cells were then washed twice with 3 ml of Ca^{++}, containing veronal buffer (18) and incubated at 37°C with a non-lytic amount of an R4 (classical serum reagent for the detection of C′4 activity), to which β_{1C}-globulin had been added. The total volume during the hemolytic reaction was 1 ml, the time of incubation varied from 5 to 45 minutes, depending upon the speed of the reaction. Lysis was terminated by addition of 2 ml ice cold veronal buffer and subsequent centrifugation when the most rapidly lysing sample in a given set had reached approximately 60 to 80 per cent lysis. The degree of lysis was determined by measuring spectrophotometrically the concentration of hemoglobin in the supernatant at 541 mμ.

Preparation of Complexes of Red Cells with Antibody and Complement.—EA (red cells coated with antibody) were prepared from sheep red cells (Probio Inc., Nyack, New York) which had been stored for several weeks in Alsever's solution by treating these cells with heat-inactivated rabbit antiserum to sheep red cells (Cappel Laboratories, West Chester, Pennsylvania) in the presence of 0.01 M EDTA. The cells were coated with two hemolysin units, which corresponded to a final dilution of antiserum of 1:2500. Thereafter the cells were centrifuged and suspended in Ca^{++} and Mg^{++} containing veronal buffer.

EAC′1 (sensitized cells containing the first component of complement, C′1) were obtained by sensitizing cells with unheated rabbit amboceptor in the presence of Ca^{++}. Even at a dilution of 1:2500 the rabbit antisera supplied a sufficient amount of C′1 to allow the formation of EAC′1 cells (19).

EAC′1-DFP refers to sensitized cells which have interacted with C′1 and which were treated with diisopropylfluorophosphonate in a final concentration of 5×10^{-3} M, at 37°C for 90 minutes (4) to inhibit C′1 esterase. After incubation the cells were thoroughly washed in Ca^{++} and Mg^{++} containing veronal buffer and utilized immediately.

EAC′1,4 cells were obtained by treatment of EAC′1 cells with an excess of highly purified β_{1E}-globulin (2 μg per 2.5 $\times 10^8$ cells in a total volume of 0.5 ml or multiples thereof). The cells were held at 37°C for 30 minutes to attain a high degree of EAC′1,4 formation. Since

[1] In experiments with complement treated immune precipitates, which were dissolved before electrophoresis in antigen excess, antigen (bovine albumin) was admixed to the agar in a concentration of 35 mg/ml.

C'1 is able to transfer to different sites (20–22), C'4 uptake can be considerable even if the amount of C'1 is limited, provided sufficient time is allowed for the reaction to proceed.

Agglutination Tests.—Antiserum to β_{1E}-globulin was inactivated by heating to 56°C for 30 minutes and thereafter six twofold serial dilutions were made beginning with a dilution of 1:10. One drop of diluted antiserum, one drop of saline, and one drop of a 1 per cent red cell suspension were mixed on a slide and after 10 minutes examined for agglutination.

Inhibition of Immune Hemolysis by Anti-β_{1E}.—Anti-β_{1E} in various dilutions was added to samples containing 2.5×10^8 EAC'1,4 cells. The volume of the reaction mixture was adjusted to 0.6 ml. After 5 minutes at room temperature, lysis was initiated by addition of R4 plus β_{1C}-globulin and by transfer of the tubes to a 37°C water bath. Inhibition of lysis was estimated by comparing the degree of lysis in samples containing anti-β_{1E} with that of a control which was devoid of antiserum. In another set of experiments the cells were washed after exposure to anti-β_{1E}, centrifuged, and resuspended in veronal buffer before lysis was initiated. Similar results were obtained with both procedures.

RESULTS

Isolation of β_{1E}-Globulin.—Since C'4 activity in human serum is associated largely with pseudoglobulins, this fraction was chosen as starting material for its purification. Exploratory experiments showed that C'4 activity adsorbed to TEAE cellulose remains adsorbed up to a sodium chloride concentration of 0.11 M, while 90 per cent of the pseudoglobulins are eluted under these

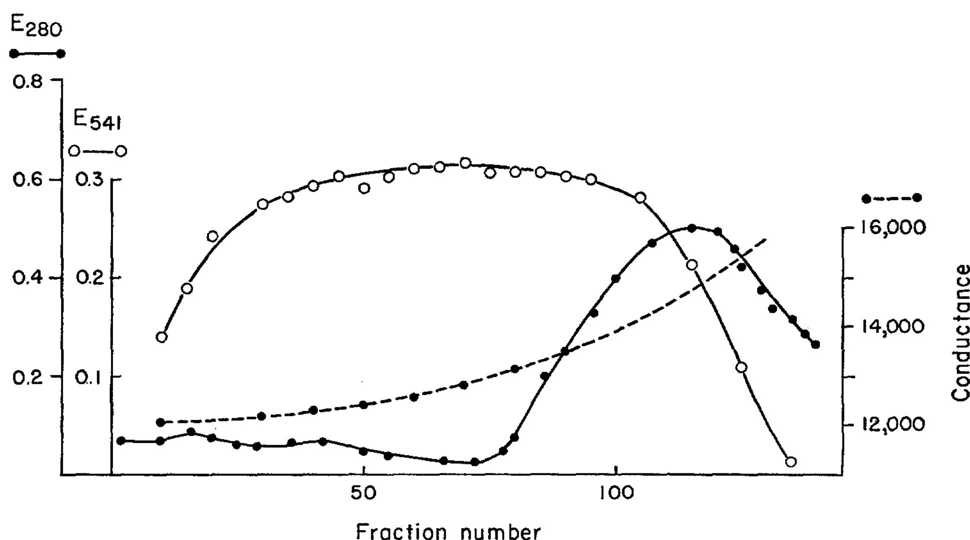


FIG. 1. Salt gradient elution of C'4 activity from pseudoglobulin fraction previously adsorbed to TEAE cellulose. After application of the protein, column was washed with 0.11 M NaCl to remove major portion of pseudoglobulin before gradient was started at fraction 1. Fractions of low protein concentration containing C'4 activity (Nos. 20 to 75) were pooled and concentrated for further electrophoretic separation.

conditions. Accordingly, in the final procedure of isolation, TEAE cellulose columns, after application of pseudoglobulin, were washed with 0.11 M NaCl in starting buffer. Thereafter, C'4 activity was eluted by gradually increasing the salt concentration. Simultaneously, collection of fractions was started. The activity was found distributed over approximately 50 fractions of very low protein content, corresponding to 500 to 700 ml of effluent (Fig. 1).

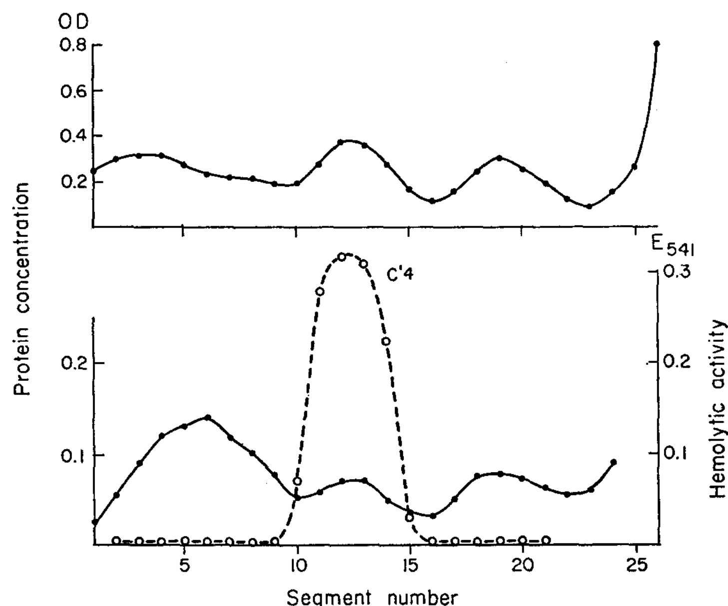


FIG. 2. Preparative zone electrophoresis on Pevikon block of C'4 activity containing column fractions (Nos. 20 to 25, Fig. 1) (lower pattern). Comparison with the electrophoretic pattern of whole serum (upper pattern) shows C'4 activity to migrate in the β-globulin region. Origin was segment 1. Fractions 11 to 14, which contained most of the C'4 activity, were pooled and concentrated for further chromatographic separation.

After concentration by ultrafiltration this material was examined immunologically and found to contain small amounts of 19S γ-globulin, ceruloplasmin, albumin, and some other proteins, notably one β-globulin. Further separation was achieved by preparative electrophoresis at pH 8.6, which afforded maximal resolution of the various proteins and which did not affect C'4 activity. As demonstrated in Fig. 2, C'4 activity has the electrophoretic mobility of a β-globulin and a relatively narrow electrophoretic distribution.

Fractions from the Pevikon block which contained C'4 activity were pooled, concentrated and once again subjected to chromatography on TEAE cellulose. A relatively shallow salt gradient was started immediately after application of the protein. Originally, this was done to check chromatographic homogeneity

of the purified material. When the result showed that a small amount of inactive protein was separated from a major active fraction (Fig. 3), rechromatography was incorporated in the isolation procedure as the final step. A summary of the entire procedure according to which more than 15 different preparations have been made, is given in Table I.

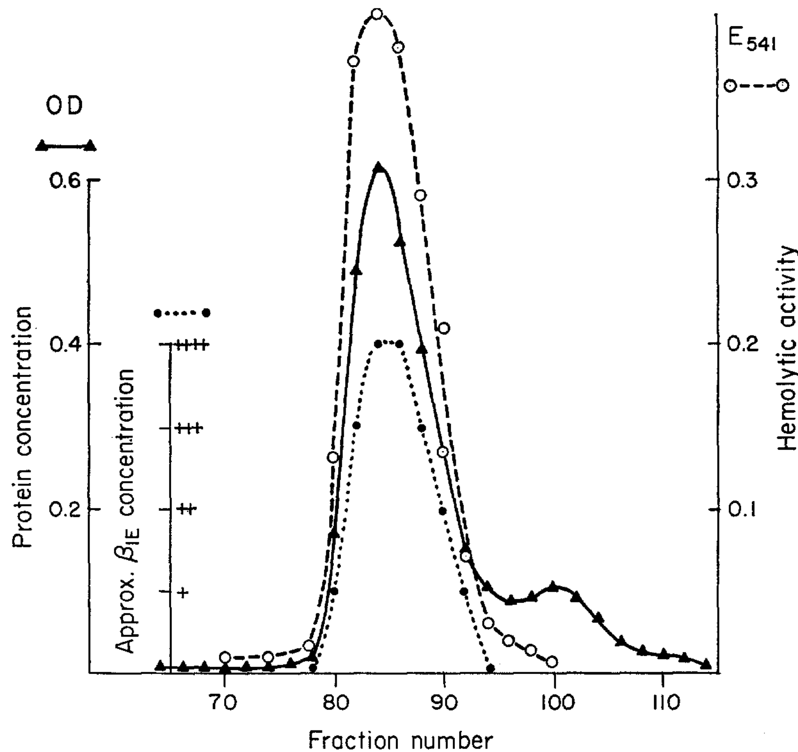


FIG. 3. Segment of chromatogram of C′4 activity containing electrophoretic fractions (Nos. 11 to 14, Fig. 2). Elution from TEAE cellulose was done by salt gradient. Note correspondence of C′4 activity with major protein fraction and with distribution of immunologically estimated β_{1E}-globulin. Fractions 80 to 92 were pooled and concentrated for further analysis.

Examination of the protein corresponding to the major fraction in Fig. 3 by immunoelectrophoresis, revealed a single component although a potent antiserum to whole human serum was used for the development of the pattern (Fig. 4). This component, which migrates in the β-globulin region, could not be identified with any of the previously described β-globulins, which have been denoted by subscripts A (10), B (23), C (10), and D (24). The new protein was therefore named β_{1E}-globulin.

Characterization of β_{1E}-Globulin.—To establish immunologic homogeneity various preparations of β_{1E}-globulin were analyzed at several different concentrations with a panel of antisera in Ouchterlony's double diffusion test. No reaction was observed with antisera to γ-globulin, β lipoprotein, and β_{1C}-

TABLE I

Procedure of Purification of C′4 Activity

1. Preparation of pseudoglobulin	Dialysis of fresh serum against PO_4 buffer pH 5.4 $\frac{\tau}{2} = 0.02$ supernatant ↓
2. Chromatography	TEAE cellulose, salt gradient (0.11 M → 1.00 M NaCl) ↓
3. Preparative electrophoresis	Pevikon block; barbital buffer, pH 8.6 $\frac{\tau}{2} = 0.05$ ↓
4. Chromatography	TEAE cellulose, salt gradient (0 → 0.5 M NaCl)

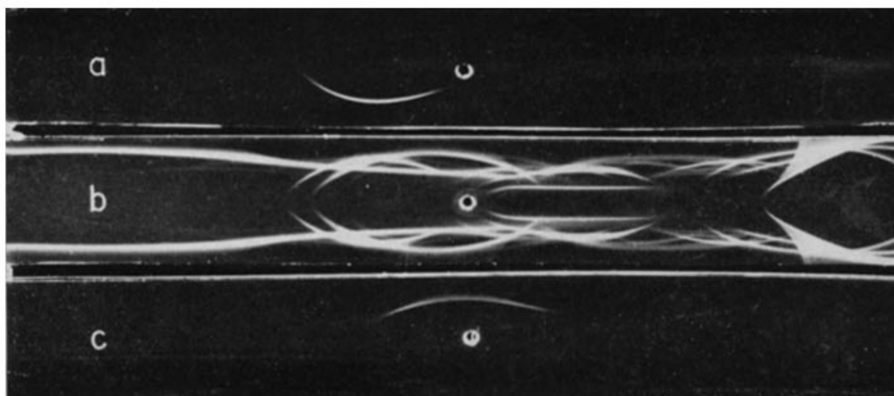


FIG. 4. Immunoelectrophoretic analysis of the final material resulting from the purification of C′4 activity (Nos. 80 to 92, Fig. 3). A single precipitin line is seen (*c*); the corresponding protein was called β_{1E}-globulin. For comparison the patterns of whole serum (*b*) and of purified β_{1C}-globulin (*a*) are shown. The antiserum used was directed to whole human serum and prepared in rabbits (Behringwerke A. G.).

globulin. A single precipitin line was obtained with each of two different antisera to whole human serum (No. 256B of Behringwerke, A. G., and No. 13415 of Institute Pasteur). Antisera to isolated β_{1E}-globulin also gave rise to one precipitin line only and this line could be shown to be identical with that produced by antisera to whole human serum. These results indicate that prep-

arations of β_{1E}-globulin can be obtained in a high degree of immunologic homogeneity (Fig. 5).

That β_{1E}-globulin has a lower diffusion rate and, perhaps, a higher molecular weight than γ-globulin was suggested by the appearance of its precipitin line in the Ouchterlony test. As seen in Fig. 5, the line is slightly concave toward the antigen well. Ultracentrifugation disclosed molecular homogeneity (Fig. 6), and from the s rate *vs.* concentration plot (Fig. 7), the corrected sedimentation coefficient, $s^{o}_{20,w}$ was obtained and found to be 10.0S. This plot showed that

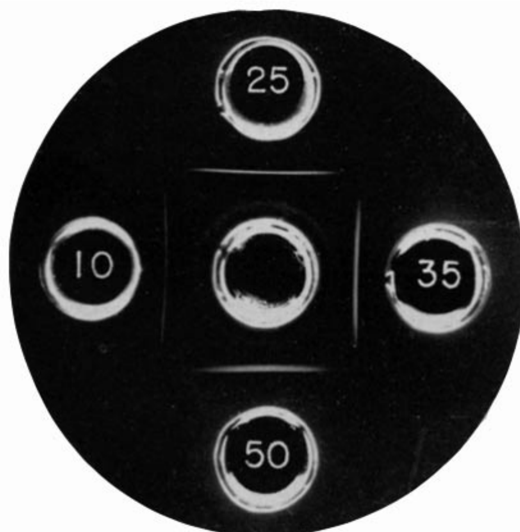


FIG. 5. Analysis of various amounts (μg) of purified β_{1E}-globulin by Ouchterlony's double diffusion test, using antiserum against whole human serum (center well). Only one precipitin line developed which is characterized by being slightly concave toward the antigen well. An identical result was obtained with a specific antiserum to β_{1E}-globulin.

the sedimentation velocity of β_{1E} is virtually independent of protein concentration which is indicative of a rather spherical shape of the molecule.

Comparison of PhysicoChemical Properties of β_{1E}-Globulin and of C′4 Activity.—Having purified β_{1E}-globulin in the course of the isolation of C′4 activity, the question arose whether C′4 activity is a function of the β_{1E} molecule or whether there is no relationship at all. A number of experiments were carried out to examine the degree of correlation between β_{1E} and C′4. Three physicochemical parameters were studied; electrophoretic mobility, sedimentation, and chromatographic behavior. As shown in Fig. 3, the chromatographic distribution of C′4 activity was found to correspond very closely to that of β_{1E}-globulin. The latter was determined immunologically by means of a specific

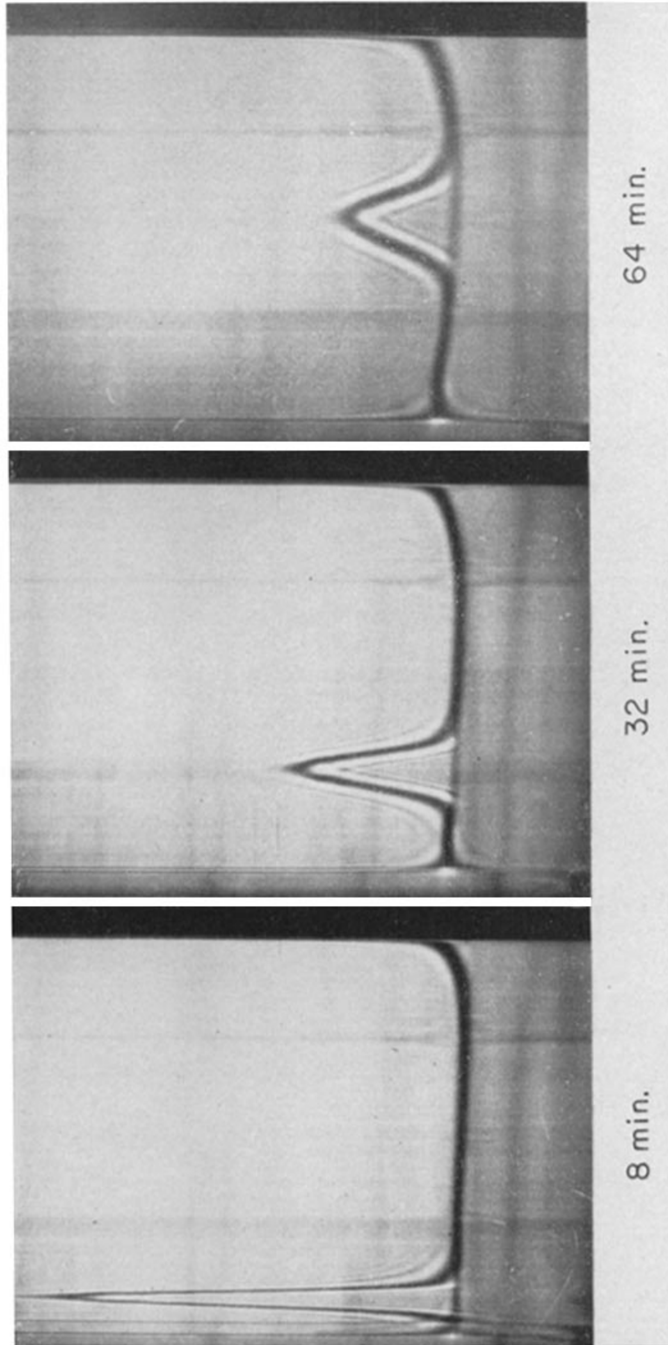


FIG. 6. Schlieren patterns of isolated β_{IE}-globulin photographed at different times during ultracentrifugation.

antiserum. Fig. 8 demonstrates the sedimentation characteristics of C′4 activity in relation to β_{1E}. A highly purified preparation of β_{1E} was subjected to zone ultracentrifugation in a sucrose density gradient. The activity exhibited the same sedimentation velocity and distribution as the protein which sedimented as a single component. For electrophoretic analysis starch gel electrophoresis was selected because this method allows separation of proteins not only according to their charge but also according to size and shape. Again, the activity was found in the position of the protein which had migrated through

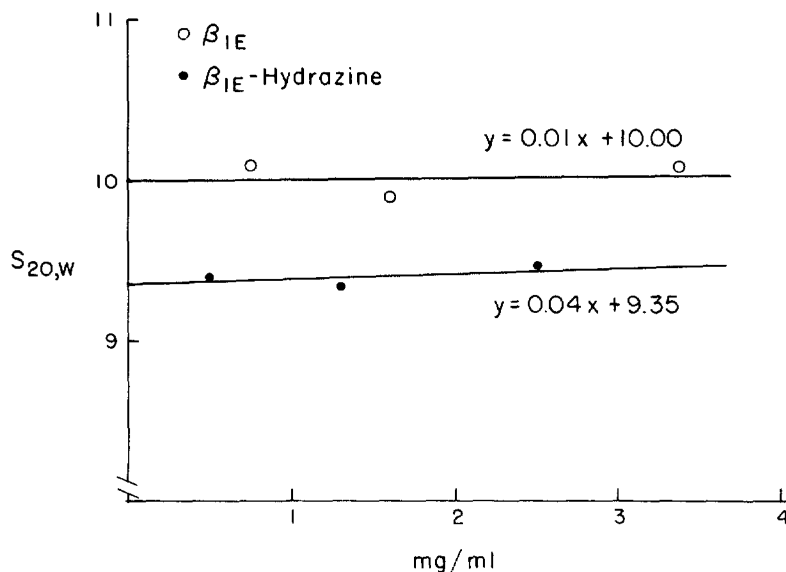


FIG. 7. s *vs.* c plot, showing virtual lack of concentration dependence of s rate of β_{1E}-globulin. Treatment with 0.015 M hydrazine at 37°C for 60 minutes results in small decrease of sedimentation velocity. y was obtained by the least square method.

the gel as a single zone (Fig. 9). These results suggested that the activity is a function of β_{1E}.

Uptake of β_{1E}-Globulin by EAC′1 cells.—In order to explore further the possibility of identity between β_{1E} and C′4 the behavior of β_{1E} during the first steps of the complement reaction was studied. It is characteristic for C′4 activity that uptake by sensitized cells requires the presence of activated C′1. If C′1 is absent or if its esterase activity has been blocked by prior treatment with DFP, C′4 activity is unable to combine with cells. This well established fact is illustrated in Table II, which records the results of an experiment utilizing three batches of differently treated red cells, EA, EAC′1, and EAC′1-DFP. Each batch was exposed at 37°C to an identical amount of purified β_{1E}, was

then thoroughly washed and examined for the presence of C′4 activity and for β_{1E}. Only EAC′1 cells were converted by this treatment to the EAC′1,4 state. And only these cells could subsequently be agglutinated by a specific antiserum to β_{1E}. This shows firstly that during the interaction of complement with

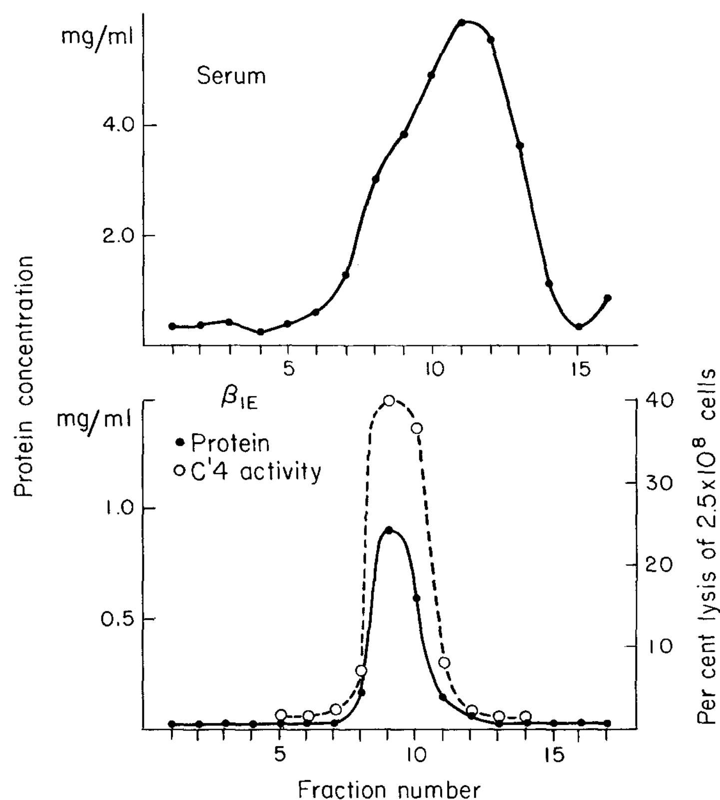
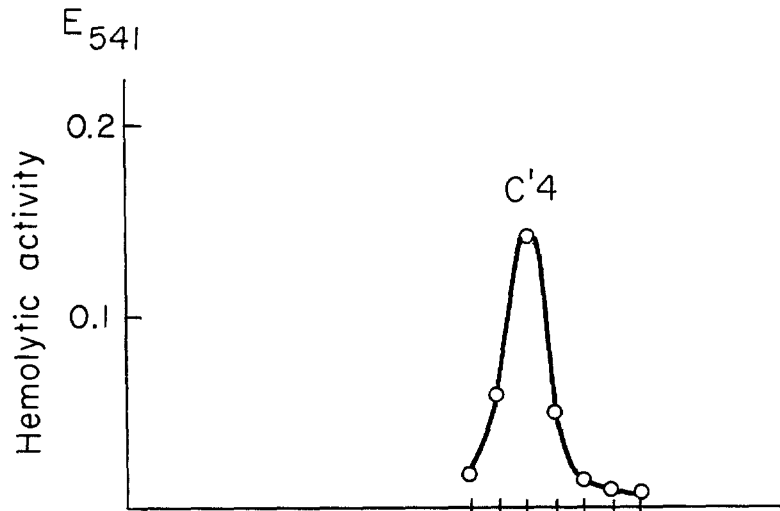


FIG. 8. Correlation between the distribution of C′4 activity and β_{1E}-globulin upon ultracentrifugation in a sucrose density gradient. Lower pattern shows that C′4 activity has a sedimentation behavior identical with that of β_{1E}. The activity curve was obtained after 2000-fold dilution of the protein fractions. For comparison the pattern of whole human serum is shown above. Direction of sedimentation is to the left.

sensitized cells β_{1E} is physically taken up and retained and secondly, that uptake, in complete analogy to C′4 activity, requires the catalytic action of a DFP-inhibitable factor, most likely C′1 esterase.

Inhibition of Lysis of EAC′1,4 Cells by Anti-β_{1E}.—Antibody to hemolytic complement components have been shown to exert an inhibitory effect on immune hemolysis (9). Rabbit anti-β_{1E} was, therefore, tested for the ability



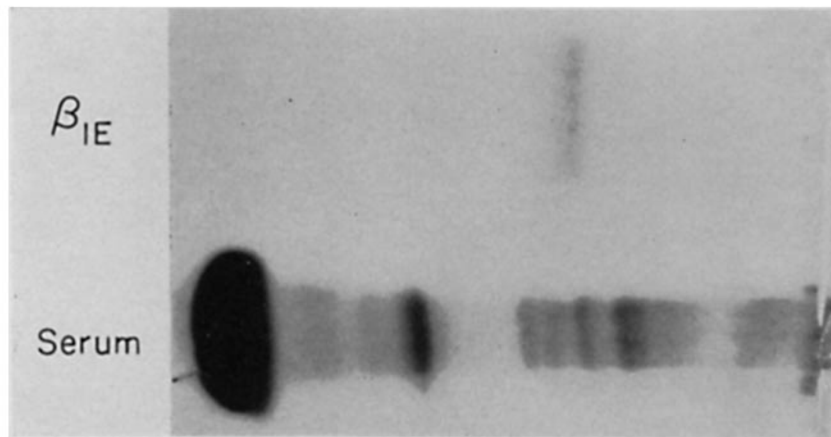


FIG. 9. Correlation of the distribution of C'4 activity and of β_{1E}-globulin upon starch gel electrophoresis. C'4 activity was found to occupy the same position in the gel as the protein zone representing isolated β_{1E}-globulin. Origin was at the right-hand edge. After electrophoresis the gel was sliced into two halves, one was stained for protein, the other served to elute the activity by freezing and thawing of small segments.

to interefere with lysis of EAC'1,4 cells. Cells of this type were exposed to anti-β_{1E} of various dilutions for a short period prior to initiation of lysis by the second and third component. As recorded in Table III, the antiserum showed a marked inhibitory effect. This effect was virtually undiminished when the EAC'1,4 cells after exposure to anti-β_{1E} were thoroughly washed before their

lysability was determined. Whether the inhibition by anti-β_{1E} is due to blocking of functional groups on the C'4 molecule or simply to sterical hindrance of subsequent complement components is presently unknown.

Demonstration of β_{1E}-Globulin in Complement-Treated Immune Precipitates.— To find out whether β_{1E} is incorporated into immune precipitates during treatment with human complement, such precipitates, after thorough washing, were dissolved in antigen excess and studied on Ouchterlony plates and by immunoelectrophoresis. With the aid of a specific antiserum, β_{1E} could invari-

TABLE II

Uptake of C'4 Activity and of β_{1E} by Cells Treated with Purified β_{1E}-Globulin

Type of cells treated with β_{1E}*	Yield of EAC'4‡	Agglutination by anti-β_{1E}					
		1:10	1:20	1:40	1:80	1:160	1:320
	per cent						
EA	0.2	0	0	0	0	0	0
EAC'1	100	3	3	3	3	3	3
EAC'1-DFP	0.2	0	0	0	0	0	0

* 2.75×10^8 cells + 9 μg β_{1E} in 0.5 ml, 30 minutes at 37°C.

‡ Tested with R4 + β_{1C}.

TABLE III

Inhibition of Lysis of EAC'1,4 Cells by Anti-β_{1E}

Final dilution of anti-β_{1E}	—*	1:1600	1:800	1:400	1:200	1:100	1:50
Per cent lysis of 2.5×10^8 EAC'1,4	64	50	34	24	9	4	2
Per cent inhibition of lysis	0	21	45	62	86	93	96

* No antiserum added.

ably be detected in these precipitates, provided incubation with complement had been carried out in the presence of bivalent cations. In the absence of bivalent cations, β_{1E} was not incorporated (Fig. 10). The amount of β_{1E} taken up was found to vary with the temperature of incubation. At 37°C, uptake was greater than at 0°C. Whether β_{1E} was completely released upon dissolution of the precipitates or whether it remained bound to soluble complexes, could not be decided on the basis of these experiments. To prevent formation of larger complexes and reprecipitation upon application to the gel, antigen in high concentration was admixed to the agar used for diffusion plates and immunoelectrophoresis. Upon immunoelectrophoresis of dissolved precipitates, β_{1E} was found to have a somewhat greater mobility than in the control serum. This may indicate that it was bound to complexes, particularly since the antigen

in these experiments was bovine albumin which has a high electrophoretic mobility. However, it could also indicate a change in physicochemical properties of β_{1E} which may have occurred during the reaction between antigen-antibody aggregates and complement. On diffusion plates the "released" β_{1E} gave the phenomenon of immunological identity with the original protein in the control serum.

Effect of Hydrazine on β_{1E}-Globulin.—Since hydrazine destroys C′4 activity (25), it was of interest to see whether it has a readily detectable effect on

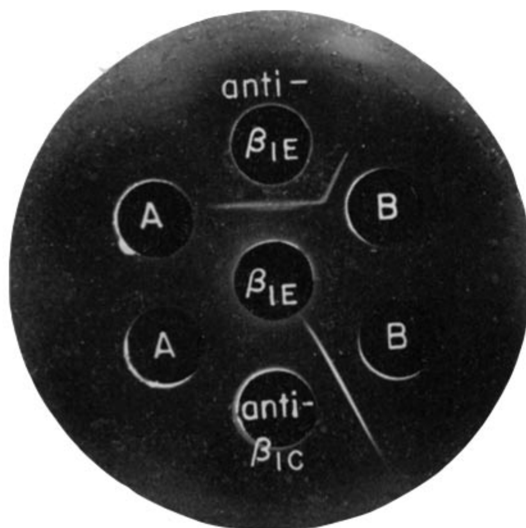


FIG. 10. Ouchterlony plate, demonstrating specific incorporation of β_{1E}-globulin from fresh human serum into immune precipitates. Antigen-antibody precipitates previously treated with human serum for 1 hour at 37°C either in the presence of 0.01 M EDTA (*A*) or in the presence of Ca^{++} and Mg^{++} (*B*) were thoroughly washed, dissolved in antigen excess, and examined with anti-β_{1E}, as well as with anti-β_{1C}. Both proteins can be seen to be present only in the precipitate which was allowed to react with complement.

β_{1E}-globulin. Table IV records the observation that after hydrazine treatment β_{1E} has not only lost C′4 activity but also the ability to combine with EAC′1 cells. The sedimentation velocity in the ultracentrifuge appeared to be somewhat diminished after hydrazine treatment (Fig. 7). The $s^{o}_{20,w}$ was 9.4S as compared to 10.0S of the untreated protein. There was no loss of antigenic determinants detectable when treated and untreated β_{1E} were tested on agar diffusion plates against anti-β_{1E}. The immunoelectrophoretic analysis of the protein after various times of exposure to hydrazine at 37°C disclosed a more drastic change of properties. As illustrated in Fig. 11, this treatment results first in an increased electrophoretic mobility and after a few minutes in a

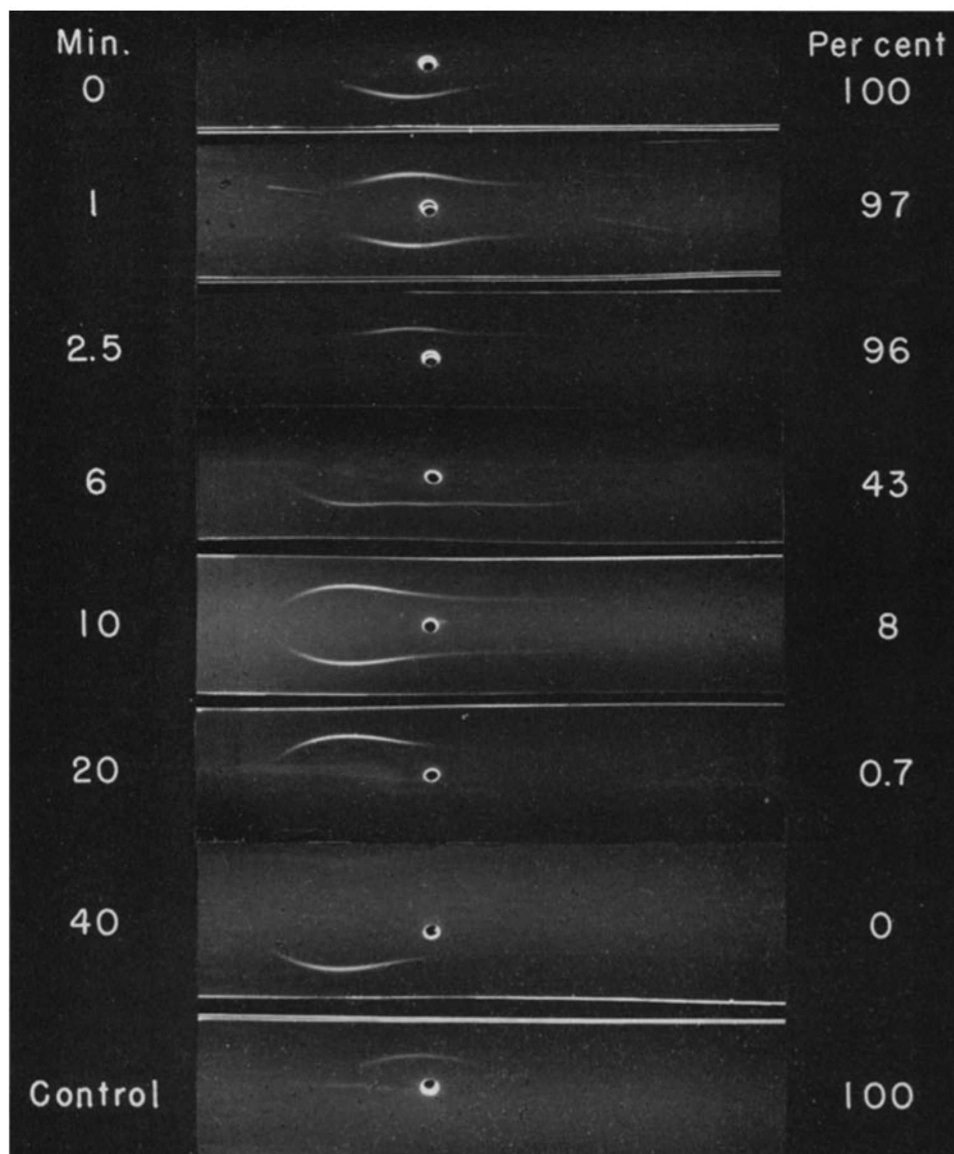


FIG. 11. Immunoelectrophoretic analysis of β_{1E}-globulin at various times during treatment with 0.015 M hydrazine at 37°C. The times at which the samples were withdrawn and placed in an ice bath are indicated on the left. The percentage of residual C′4 activity is listed on the right. The anode was on the right-hand side.

TABLE IV

Effect of Hydrazine upon Uptake of β_{1E}-Globulin by EAC'1 Cells

2.7×10^8 cells incubated with 5 μg of	Yield of EAC' 1,4	Agglutination by anti-β_{1E}					
		1:10	1:20	1:40	1:80	1:160	1:320
	per cent						
β_{1E}	100	3	3	3	3	2	0
β_{1E}-hydrazine*	0	0	0	0	0	0	0

* Incubated at 37°C for 60 minutes with 0.01 M hydrazine at pH 7.3.

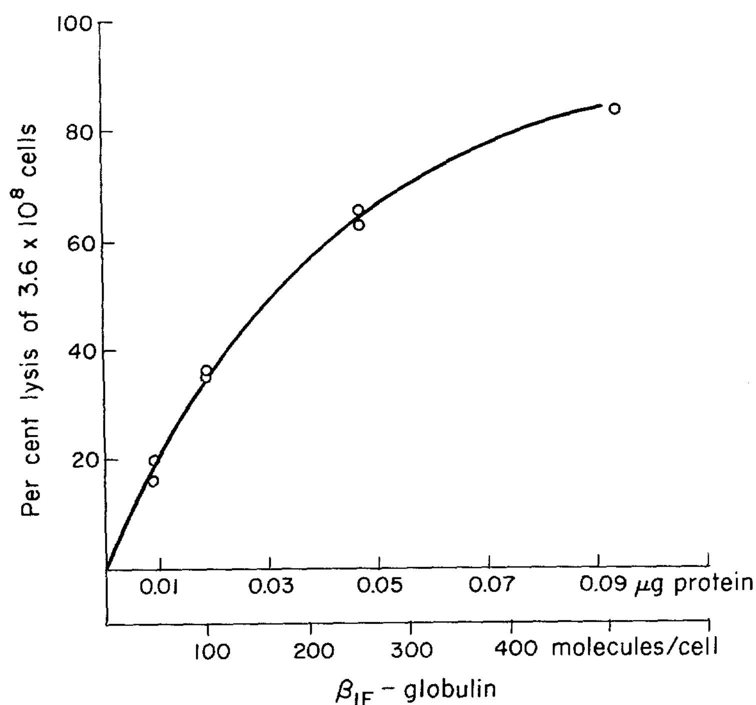


FIG. 12. Quantitative relation between β_{1E}-globulin offered to EAC'1 cells and the formation of EAC'1,4 cells.

marked decrease of mobility, so that the final product migrates more slowly than the control. The sample taken at 6 minutes of incubation shows clearly the presence of three molecular species differing in mobility, the original material, and the faster and slower product. Loss of C'4 activity appears to correspond to the appearance of the slow product. The chemical reaction underlying these mobility changes is presently unknown. According to Seifter *et al.* (26) the reaction conditions suggest a nucleophilic attack on an ester.

Quantitative Relation between β_{1E} and the Formation of EAC′1,4 cells.—Fig. 12 shows a dose response curve which reflects the extent of uptake of C′4 activity by EAC′1 cells from various amounts of β_{1E}-globulin. The quantity of β_{1E} required to convert a large proportion of 360 million cells to the EAC′1,4 state is of the order of a few hundredth of a microgram, which corresponds to a few hundred molecules per cells. At 63 per cent lysis, for instance, approximately 250 molecules per cell are needed. It is important to note that this figure indicates the number of molecules which were offered to the cells but not the number of molecules which were actually taken up. Preliminary results with I-131–labeled β_{1E} show that only a fraction of the protein is taken up by EAC′1 cells during a given period of incubation. Furthermore, it was found that binding also occurred with cells lacking the first component and that the protein thus bound was hemolytically ineffective. The amount of β_{1E} that was attached, specifically through the catalytic action of C′1, was therefore much smaller than the total amount bound. Thus the number of molecules effecting a certain degree of lysis is considerably smaller than indicated in Fig. 12. These quantitative aspects are presently under investigation and details will be reported in a later communication.

DISCUSSION

As has been shown above, purification of C′4 activity resulted in the isolation of a physicochemically and immunologically homogeneous protein. The primary concern of this study was to investigate whether the new protein is only a by-product in the purification of C′4 activity or whether it actually represents the fourth component of complement. Examination of the two entities with respect to physicochemical characteristics revealed a striking resemblance. Virtually identical distribution patterns were obtained for both upon chromatography on TEAE cellulose, ultracentrifugation in a sucrose density gradient, preparative electrophoresis on Pevikon, and electrophoresis in starch gel. This failure to separate activity and protein by a variety of powerful techniques strongly suggested that C′4 activity is a function of the β_{1E} molecule.

Nevertheless, the possibility remained that the activity is carried by molecules which are completely unrelated although physicochemically similar to β_{1E}-globulin. If these molecules constituted only a small proportion of a given β_{1E} preparation, they might conceivably escape detection even by immunologic techniques. It therefore became necessary to examine the correlation between activity and protein under application of more specific criteria. Thus the behavior of β_{1E} in immune reactions was studied and compared with the known behavior of C′4 activity. These experiments disclosed that β_{1E} is incorporated into immune precipitates during their incubation with fresh human serum and, more important, is taken up and firmly bound by cells which contain

activated C′1 esterase. Uptake is catalyzed by a DFP-inhibitable factor on the cells, probably C′1 esterase. Moreover, hydrazine treatment which effected certain changes in the physicochemical properties of the protein, rendered it incapable of combining with C′1-containing cells. Therefore, the conclusion was inescapable that β_{1E} is identical with the fourth component, which thus is the first of the classical components to be immunologically identified and to be obtained in highly purified form.

Partial purification of C′4 of guinea pig serum has previously been achieved by Hoffman (21), who was able to eliminate from a C′4 containing serum fraction virtually all other complement component activities. With such preparations, Hoffman established that the action of C′4 is stoichiometric in the sense that the number of effective C′4 sites formed on EAC′1,4 cells is proportional to the amount of C′4 added. Similarly, if the β_{1E} dose response curve shown in Fig. 12 is expressed in terms of C′4 sites produced instead of in percent lysis, a nearly linear function is obtained.

Of considerable interest is the question as to the proportion of molecules in β_{1E} preparations which is capable of interacting with C′1-containing cellular sites. As illustrated in Fig. 12, approximately 250 molecules per cell were needed to achieve 63 per cent lysis. Preliminary experiments with I-131-labelled β_{1E} indicate that only a certain percentage of these molecules combined with the cells. What happened to the molecules which remained in the fluid phase? Were these inactive analogues, incapable of combining with EAC′1 cells or were these acted upon by C′1 in a side reaction as postulated by Mayer (27), which resulted in inactivation instead of in uptake? Inactivation of β_{1E} by C′1 is not an improbable side reaction to occur since Lepow and Pillemer (8) showed that activated C′1 esterase can destroy both, C′4 and C′2 activity. On the other hand it is not improbable either that a certain proportion of molecules is rendered inactive by denaturation during isolation.

A more difficult problem is the question of whether all β_{1E} molecules which are taken up specifically through the catalytic action of C′1 are hemolytically effective. The β_{1E} molecule possesses probably more than one site critical for its function. Through one of these it combines with the cell, whereas another site might be essential for interaction with C′2 or even C′3. The former site can be destroyed by hydrazine, since after such treatment β_{1E} has lost the ability to combine with EAC′1. Hydrazine, however, has no effect on the other postulated site since treatment of EAC′1,4 cells with hydrazine neither inactivates cell-fixed C′4 activity nor splits off β_{1E}. It is conceivable therefore, that handling of the protein during purification might affect the postulated second site of some of the molecules but not the combining site, which would allow specific uptake but bar hemolytic function.

It will be most interesting to elucidate the identity of the receptor for β_{1E} and the nature of the bond by which β_{1E} is attached to the cell complex, and

the effect of C′1 on β_{1E}. Since C′1 can be eluted from EAC′1,4 cells without causing release of C′4 (4), C′1 cannot be the receptor, for β_{1E}. Receptor can, therefore, only be the antibody, sensitizing the cell, or the cell membrane itself. Since uptake of β_{1E} is catalyzed by an enzyme, the bond between protein and receptor is probably a covalent one. Because of the enzymatic activity of C′1, action of C′1 on β_{1E} should result in distinct changes of the properties of the protein. Experiments to detect such changes are presently in progress.

SUMMARY

Purification of the activity of the fourth component of human complement resulted in the isolation of a highly homogeneous serum protein. Since this protein has not been recorded previously it was called β_{1E}-globulin on the basis of its immunoelectrophoretic behavior. C′4 activity and β_{1E}-globulin were found to have highly similar, if not identical physicochemical characteristics. Moreover, β_{1E}-globulin was shown to exhibit the specific behavior of C′4 activity in that it is taken up only by cells which contain activated C′1. DFP-inactivated C′1 failed to catalyze uptake of the protein. Treatment with hydrazine which is known to destroy C′4 activity, led to changes in the physicochemical properties of β_{1E}-globulin and rendered the molecule incapable to combine with C′1-containing cells. The evidence indicates that β_{1E}-globulin represents the fourth component of human complement.

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BIBLIOGRAPHY

1. Heidelberger, M., A quantitative, absolute method for the estimation of complement (Alexin), *Science*, 1940, **92,** 534.
2. Gordon, J., Whitehead, H. R., and Wormall, A., The action of ammonia on complement. The fourth component, *Biochem. J.*, 1926, **20,** 1028.
3. Laporte, R., de Looze, L. H., and Sillard, R., Contribution a l'étude du complément. II. Premier stade de l'action hémolytique du complément, *Ann. Inst. Pasteur*, 1957, **92,** 15.
4. Becker, E. L., Concerning the mechanism of complement action. V. The early steps in immune hemolysis, *J. Immunol.*, 1960, **84,** 299.
5. Klein, P. G., Studies on immune hemolysis: Preparation of a stable and highly reactive complex of sensitized erythrocytes and the first component of complement (EAC′1); Inactivation of cell-fixed C′1 by some complement reagents, *J. Exp. Med.*, 1960, **111,** 77.
6. Pillemer, L., Seifter, S., Chu, F., and Ecker, E. E., Function of components of complement in immune hemolysis, *J. Exp. Med.*, 1942, **76,** 93.
7. Becker, E. L., Inhibition of complement activity by di-isopropyl-fluorophosphate, *Nature*, 1955, **176,** 1073.
8. Lepow, I. R., Ratnoff, O. D., Rosen, F. S., and Pillemer, L., Observations on a

pro-esterase associated with partially purified first component of human complement (C′1), *Proc. Soc. Exp. Biol. and Med.*, 1956, **92,** 32.
9. Mayer, M. M., Summary of complement workshop, *Science*, 1963, in press.
10. Müller-Eberhard, H. J., Nilsson, U., and Aronsson, T., Isolation and characterization of two β_1-glycoproteins of human serum, *J. Exp. Med.*, 1960, **111,** 201.
11. Harboe, M., Müller-Eberhard, H. J., Fudenberg, H., Polley, M., and Mollison, P. L., Identification of the components of complement participating in the antiglobulin reaction, *Immunology*, 1963, **6,** in press.
12. Müller-Eberhard, H. J., Isolation and description of proteins related to the human complement system, *Acta Soc. Med. Upsaliensis*, 1961, **66,** 152.
13. Müller-Eberhard, H. J., A new supporting medium for preparative electrophoresis, *Scand. J. Clin. and Lab. Inv.*, 1960, **12,** 33.
14. Scheidegger, J. J., Une micro-méthode l'immuno-éléctrophorese, *Internat. Arch. Allergy*, 1955, **33,** 11.
15. Smithies, O., Zone electrophoresis in starch gels and its application to studies of serum proteins, *Advances Protein Chem.*, 1959, **14,** 65.
16. Poulik, M. D., Starch gel electrophoresis in a discontinuous system of buffers, *Nature*, 1957, **180,** 1477.
17. Kunkel, H. G., Macroglobulins and high molecular weight antibodies, *The Plasma Proteins*, 1960, **1,** 279.
18. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1961, chapter 4, 133.
19. DeLooze, L. L., Ransby, A., Leon, M., Activity of C′1 in rabbit anti-sheep hemolysin, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109,** 393.
20. Becker, E. L., Early steps in immune hemolysis, *in* Immunochemical Approaches to Problems in Microbiology, New Brunswick, New Jersey, Rutgers University Press, 1961, 245.
21. Hoffmann, L. G., Purification of the first and fourth component of guinea pig complement and studies on their mechanism of action, Dissertation, The Johns Hopkins University School of Hygiene and Public Health, 1960.
22. Borsos, T., and Mayer, M. M., Mechanism of action of guinea pig complement, *in* Mechanism of Cell and Tissue Damage Produced by Immune Reactions, (P. Grabar and P. Miescher, editors), Basel, Benno Schwabe and Co., 1962, 13.
23. Grabar, P., De Vaux St.-Cyr, C., and Cleve, H., Presence de β_{1B}-globuline dans les extraits perchloriques de serums humains normaux, *Bull. Soc. Chim. biol.*, 1960, **42,** 853.
24. Williams, C. A., and Wemyss, C. T., Changes produced in mouse plasma proteins by acute bacterial infections, *J. Exp. Med.*, 1961, **114,** 311.
25. Ecker, E. E., Pillemer, L., and Seifter, S., Immunochemical studies on human serum. I. Human complement and its components, *J. Immunol.*, 1943, **47,** 181.
26. Seifter, S., Katchalski, E., and Harkness, D. M., Effects of hydroxylamine on complement, *Fed. Proc.*, 1963, **22,** 612.
27. Mayer, M. M., On the role of complement and its mechanism of action, 4th Michael Heidelberger Lecture, 1963.