

THE ANTIGENIC AND MOLECULAR ALTERATIONS OF C3 IN  
THE FLUID PHASE DURING AN IMMUNE REACTION  
IN NORMAL HUMAN SERUM

DEMONSTRATION OF A NEW CONVERSION PRODUCT, C3x\*

BY R. E. SPITZER, M.D., A. E. STITZEL, V. L. PAULING, N. C. DAVIS, PH.D.,  
AND C. D. WEST, M.D.

*(From The Children's Hospital Research Foundation and the Department of Pediatrics,  
University of Cincinnati College of Medicine, Cincinnati, Ohio 45229)*

(Received for publication 8 March 1971)

The details of the reaction mechanism of C3 have been extensively investigated in studies employing pure complement components and sensitized erythrocytes (1, 2). The dynamics of these reactions have been followed by radioisotope labels and the end products detected and identified by their sedimentation and electrophoretic characteristics. More recent studies (3) have focused on the reaction of C3 with certain enzymes such as trypsin, thrombin, plasmin, or the C3 inactivator complex formed from cobra venom and a serum beta globulin. Again these investigations have utilized purified substances and the resulting products have been analyzed by many of the same techniques used in earlier studies.

The present investigation of the reaction sequence of C3 differs from those described above in that (a) an immune precipitate has been substituted for sensitized erythrocytes, (b) the reaction has been carried out in fresh human serum rather than with purified complement components, and (c) monospecific antisera to the antigenic determinants of C3 (4) have been used to study the breakdown products formed. The use of serum approximates *in vivo* conditions more closely than is possible with purified components, a feature which becomes important in assessing complement reactions in disease. The use of monospecific antisera has added an additional parameter to the characterization of the C3 conversion products and has allowed positive identification of these products after electrophoresis in acrylamide or agar, despite the presence of contaminating serum proteins. The present paper deals primarily with the identification of the C3 conversion products as they occur in the fluid phase during the reaction of fresh human serum with an immune precipitate. In addition, the kinetics of this fluid-phase reaction have been studied. Subsequent communications

\* This investigation was supported by U. S. Public Health Service Research Grant No. AM-14413, General Research Support Grant No. 5-SO1-RR-05535, and by National Institutes of Health Special Fellowship No. 5-F03-AM-36243 (Dr. R. E. Spitzer).

will deal with the qualitative and quantitative aspects of the bound phase, i.e., the deposition of C3 on the immune complex.

### *Materials and Methods*

*Specific Antisera to A, B, and D Antigens of C3.*—To produce antibody to B and D determinants of C3, goats were immunized with purified C3 (5) incorporated in complete Freund's adjuvant. Approximately 1 mg was given at each of 10 subcutaneous sites at each of three immunizations 7–10 days apart. Starting on the 5th day after the third immunization, the animal was bled of 200 ml daily until the 12th day. During this period, antibody titers to D and B antigens reached a peak and then fell off. Since peak titers are present for only a short period, daily bleedings are necessary (4). The response of the animals to the immunization varied: some developed antibody only to the A antigen, some to the A antigen as well as D or B antigens, and some to all three antigens.

Antiserum specific for the B determinant was made by absorbing appropriate antiserum raised against C3 with aged serum. The aged serum removes antibody to A and D antigens.

Antiserum specific for the D determinant must start with one raised against C3 containing little or no anti-B. This antiserum is absorbed with aged serum heated to 56°C for 1 hr. The heating destroys the D antigenic determinant but does not affect the A determinant.

Antiserum containing antibody only to the A antigen was made by immunizing as above with purified  $\beta$ 1A globulin. Antibody to the A antigen persists for a much longer time in the immunized animal than does that to the other two determinants so that the time of bleeding is not critical.

All antisera were checked for specificity by using them for immunoelectrophoretic analysis of fresh, aged, and partially aged serum. All antisera were monospecific in terms of containing antibody to only one antigenic determinant of C3. Some of the antisera used in this study, however, contained trace amounts of antibody to serum proteins other than C3. These were not removed as their presence did not interfere with the interpretation of the immunoelectrophoretic patterns. For those studies using immunopolyacrylamide gel electrophoretic analysis, the antisera were absorbed so that they contained antibodies to C3 alone.

*Agar Immunoelectrophoretic Analysis.*—The micromethod of Scheidegger was used (6). Disodium ethylenediaminetetraacetic acid (EDTA) was added to the agar, 18.6 mg/5 ml of agar.

*Preparation of Immune Precipitates.*—Goats were immunized with purified crystalline bovine serum albumin (BSA)<sup>1</sup> obtained from Pentex Biochemical, Kankakee, Ill. The BSA was incorporated in complete Freund's adjuvant to a final concentration of 6 mg/ml and injected subcutaneously, 1 ml being given at each of 10 sites at each immunization. Goats were immunized at weekly intervals for 3 wk and then bled of 800 ml on the 5th and 7th days after the third immunization. The equivalence point of the antiserum was determined by the disappearance of antibody after addition of increasing quantities of BSA. Antibody disappearance was detected by using the supernatants of the mixtures to develop the precipitin arc for BSA electrophoresed in agar (7).

The immune precipitates were prepared by adding BSA at equivalence to the antiserum after it had been heated to 56°C for 1 hr. The mixture was then diluted with 0.15 M NaCl in the amount of 10 ml/ml of antiserum. After 30 min of incubation at 37°C, the immune precipitate was allowed to stand at 4°C for 16 hr. The precipitate was then centrifuged in the cold, washed several times with cold 0.15 M sodium chloride, and used within a few hours.

The goat anti-BSA-BSA immune precipitates were also prepared without heating the antisera but in the presence of 0.02 M EDTA. In addition, in order to prevent a complement reac-

<sup>1</sup> Abbreviations used in text: BSA, bovine serum albumin; NHS, normal human serum.

tion during the preparation of the immune precipitates, the isolated IgG fraction of goat antisera to BSA was used as a source of antibody. To be certain that the results obtained were not merely a by-product of the use of goat antibody, antisera to BSA were also prepared in rabbits. Finally, two other antigens were utilized in the preparation of immune precipitates, an isolated fraction of human IgG, and ovalbumin. Antibody in these two cases were prepared in the goat and guinea pig respectively. Lastly, isolated and purified human IgG was aggregated by pH or heat and tested as another agent which would mediate the conversion of C3. In all cases, the kinetics of the changes in A, B, and D antigens of C3, as well as the agar and acrylamide immunoelectrophoretic patterns, were comparable.

*Normal Human Serum.*—Blood was obtained from healthy adult male and female donors, allowed to clot at 4°C, and centrifuged at 4°C. The serum was used on the same day it was obtained.

EDTA plasma was prepared by drawing blood directly into a test tube containing 18.6 mg of EDTA/5 ml of blood.

*The Reaction of Normal Human Serum with Immune Precipitates.*—Immune precipitate was centrifuged in a calibrated centrifuge tube for 20 min at 2000 *g*, the supernatant was decanted, and the precipitate was broken up by use of a stirring rod and a vortex mixer. After the precipitate and normal human serum (NHS) had been separately warmed to 37°C, the two were mixed in a ratio of 5 volumes of NHS:2 volumes of packed precipitate and the mixture was incubated at 37°C for the desired time. The pH remained between 7.0 and 7.4 during the reaction. The reaction was stopped by immersing the tube in acetone and dry ice. The mixture was then allowed to warm to 4°C in ice and the two phases were separated by centrifuging at 4°C. By this procedure, the time interval necessary to reduce the temperature of the reaction mixture from 37° to 4°C was 6 sec (using glass centrifuge tubes). Freezing the mixtures in the acetone and dry ice had no significant effect on the conversion products or on the quantitation of their antigenic determinants. The supernatants were either directly assayed or frozen at -70°C and assayed within 24 hr. All time intervals for a single kinetic experiment were performed on the same day using freshly prepared immune precipitates and freshly drawn serum.

*Quantitation Procedure.*—The antigens of C3 were quantitated by the immunoelectrophoretic precipitin method as previously described (7). The procedure however, was modified in some instances by omitting electrophoresis of the antigen and employing simple double diffusion of antigen and supernatant in agar for detecting the end point in a manner similar to that described by Wright (8). Both the original method and the double-diffusion modification were carried out in Noble agar (Difco Laboratories, Detroit, Mich.) at pH 8.6. All antisera, whether used for the original method or for the double-diffusion modification, were standardized so that the results were comparable to those in previous publications from this laboratory.

Using this experimental procedure and the immunoelectrophoretic precipitin method or its modification for quantitation, the variation of the results on duplicate specimens was less than 2% for the A and B antigens and 4% for the D antigen.

*Acrylamide Immunoelectrophoretic Analysis.*—Acrylamide gel, 7.5%, pH 8.4, was prepared according to the method of Davis (9). Samples<sup>2</sup> of 0.1 ml, diluted with 0.15 M NaCl to contain 100–200 µg total protein, were added to the gel at room temperature and electrophoresed for 2 hr at 7.5 v and 2 ma per tube. After electrophoresis, the entire gel was completely immersed in a test tube containing monospecific antiserum to one of the three antigens of C3 and allowed to soak at room temperature for 18 hr. After removal from the antiserum the gel was soaked in 0.15 M NaCl for 18–24 hr and finally imbedded in Noble agar at pH 8.6. A current was then passed across the shortest dimension of the gel for 2 hr to remove unprecipitated serum proteins. Immune precipitates deposited around the periphery of the gel were rendered visible by

<sup>2</sup> Normal human serum, serum which had reacted with an immune precipitate, aged serum, or purified complement components and their conversion products.

soaking the gel in standard amidoschwarz stain for several hours and destaining by electrophoresis in 7% acetic acid. Control gels containing serum samples but not soaked in antisera or without serum samples but soaked in antisera retained no stain. To document the mobility of the breakdown products in the gel, either purified C3 (5) or  $\beta$ 1A (10) were treated as above and used as a comparison, or a gel untreated with antibody or stain was cut in 2–3-mm segments, each segment was eluted, and the eluted proteins were identified by immunoelectrophoretic analysis using monospecific antiserum. To obtain sufficient protein from the gel for identification by immunoelectrophoretic analysis, several gels had to be eluted and the eluates pooled. The elution was accomplished by placing each gel segment in a test tube containing 0.2 ml of Veronal-buffered saline, pH 7.4, and grinding it with a glass rod. After standing for 1 hr at room temperature the tube was centrifuged for 20 min, and the supernatants were pooled, concentrated by ultrafiltration, and subjected to immunoelectrophoretic analysis.

It is noteworthy that with electrophoresis of EDTA plasma in 7.5% acrylamide gels, each of the monospecific antisera to the three determinants of C3 produced only a single band, always with the same mobility, thus indicating that, within the limits of detection by this method, the antisera were indeed monospecific. In addition, gels electrophoresed to remove ammonium persulfate before the sample was applied gave the same relative patterns as those gels which contained ammonium persulfate. The only observable difference was that the mobility of all proteins was slightly faster when the persulfate was removed. In contrast, if purified C3 or its conversion products rather than serum were applied to the gel, the ammonium persulfate interfered. With persulfate present, an extra band often appeared which was just anodal to native C3. For this reason, when purified C3 or isolated products were used, the ammonium persulfate had to be removed before application of the sample.

Acid polyacrylamide gels, 6%, were prepared at pH 4.5 by the method of Reisfield et al. (11). Ammonium persulfate was always removed before application of the sample.

When the mobilities of proteins were compared on separate polyacrylamide gels, the samples were always electrophoresed under identical conditions, i.e. in the same run, side by side, and for the same length of time. A tracer dye, bromphenol blue, was always utilized and the distance it traveled in each gel was carefully measured as an added control. No gels were compared unless the tracer dye in each migrated the same distance.

EDTA plasmas from many normal individuals were tested as a source of C3. All gave a single, discrete band on gel with each antiserum. Although the allotypes of C3 were not determined for the human serum, it was felt that the above controls eliminated the possibility of an allotypic form of C3 being misinterpreted as a conversion product. Kinetic and immunoelectrophoretic results were not affected by the source of the serum.

*Aging of Serum.*—Normal human serum was “aged” by incubating at 37°C for 7–14 days (12). To prevent bacterial contamination, sodium azide, sodium fluoride, or chloramphenicol was added at a final concentration of  $5 \times 10^{-3}$  M. Results by all three methods of preservation were identical.

*Reaction of Trypsin with Normal Human Serum.*—Trypsin,<sup>3</sup> 0.1 ml of a solution containing 10 mg/ml, was added to 0.25 ml of normal human serum for either 60 sec at 20°C, 60 min at 37°C, or 4 hr at 37°C. The reaction was stopped in each case by adding 2 mg of soybean trypsin inhibitor and then immediately subjecting the mixture to agar or acrylamide immunoelectrophoretic analysis.

*Preparation of Purified Complement Components.*—Guinea pig C1 and C2 were “functionally purified” by a modification of methods previously described (13, 14).

Human C3 was partially purified by the method of Nilsson and Müller-Eberhard (5). The

<sup>3</sup> From bovine pancreas, type III, two times recrystallized, dialyzed, and lyophilized; Sigma Chemical Co., St. Louis, Mo.

resultant C3 product contained at least three additional proteins by acrylamide gel electrophoretic analysis, none of which was a C3 breakdown product.

*Preparation of C3i.*—EAC1<sup>GP</sup>,4<sup>Hu</sup>,2<sup>GP</sup> in a concentration of  $1 \times 10^9$  cells/ml were prepared by the method of Borsos and Rapp (15). Purified C3 in a concentration of 1.5 mg of total protein/ml was added and the mixture was incubated at 37°C for 30 min. The cells were then centrifuged and the supernatant, which contained unreacted native C3 and C3i, was concentrated 10-fold by ultrafiltration.

*Preparation of C3a and C3b.*—Trypsin, 1.0 ml of a solution containing 0.1  $\mu$ g/ml, was added to 5 ml of purified C3 containing 10  $\mu$ g of protein for 60 sec at 20°C. 1 ml of soybean trypsin inhibitor at a concentration of 0.2  $\mu$ g/ml was then added and the mixture acidified to pH 3.6 by the addition of 1 N HCl. After concentration by ultrafiltration, the mixture was passed over a  $90 \times 2.5$  cm column of Sephadex G-100 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and eluted as described by Bokisch et al. (3). Both C3a and C3b were concentrated by lyophilization. In addition, the ultrafiltrate from the above mixture before Sephadex filtration was lyophilized and found to contain small amounts of C3a.

C3a was also prepared by reacting hydroxylamine with purified C3 or euglobulin (16). Hydroxylamine hydrochloride was added to euglobulin in a 0.1 M phosphate solution, pH 6.8, to a final concentration of 0.3 M. The pH was readjusted to 6.8 by the addition of a solution of Na<sub>2</sub>HPO<sub>4</sub>. After 45 min at room temperature, 2 vol of 0.1 N ammonium acetate buffer (pH 3.6) were added and the final pH was adjusted to 3.6 by 1 N acetic acid. This mixture was then ultrafiltered and the ultrafiltrate was lyophilized, dissolved in a small volume of 0.15 M NaCl, and put on a  $1 \times 6$  cm G-25 column equilibrated with a 0.0175 M phosphate solution, pH 6.3, to remove excess salts.

Finally, C3a and C3b were formed by acidifying C3i (prepared as above) to pH 4.0 with 0.1 M HCl (17) followed by separation on acid polyacrylamide gel.

In all assays, C3a was detected after electrophoresis on polyacrylamide gel at pH 4. Identification was by reaction with antibody to the B antigen of C3. C3b was detected on both pH 4 and pH 8.7 gels and C3i only on pH 8.7 gels.

## RESULTS

*Changes in the Agar Immunelectrophoretic Characteristics and Antigenic Structure of C3 During the Reaction of Normal Human Serum with an Immune Complex.*—Native C3 may be characterized by its typical electrophoretic mobility in agar and by the presence of three major antigenic determinant groups, previously designated A, B, and D (4). Both of these characteristics are illustrated in Fig. 1. As C3 in normal human serum reacts with an immune precipitate, these characteristics change in a consistent and predictable manner. In general terms, the molecule of C3 appears ultimately to be cleaved into at least two distinct breakdown products, one bearing the A antigenic determinant, designated C3c, and the other, the D determinant, designated C3d. The B antigen, which was present on the intact molecule, disappears. These changes are shown by the immunelectrophoretic patterns of serum after reaction with an immune precipitate for 3 and 15 min (Figs. 2, 3). It appears that C3c and C3d have slightly faster mobilities than native C3. That C3c and C3d are separate and distinct proteins is indicated by the double spurring shown in Fig. 4.

It is important to point out at this time that, as shown in Fig. 5, C3d in

serum reacted with an immune precipitate and  $\alpha 2D$  present in aged serum have different appearances upon immunoelectrophoretic analysis in agar. There appears to be a greater heterogeneity of  $\alpha 2D$  suggesting two populations

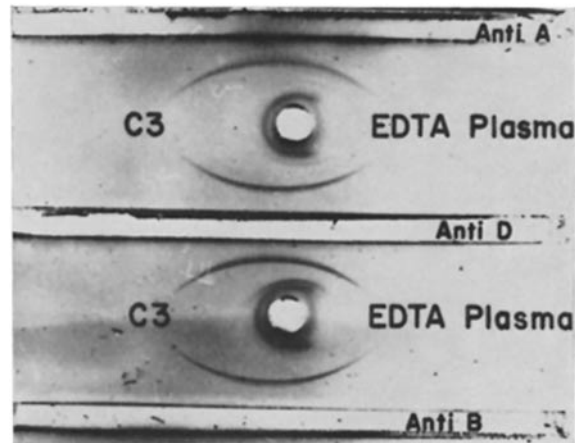


FIG. 1. Agar immunoelectrophoretic analysis of EDTA normal human plasma illustrating the three major antigenic determinants of C3.

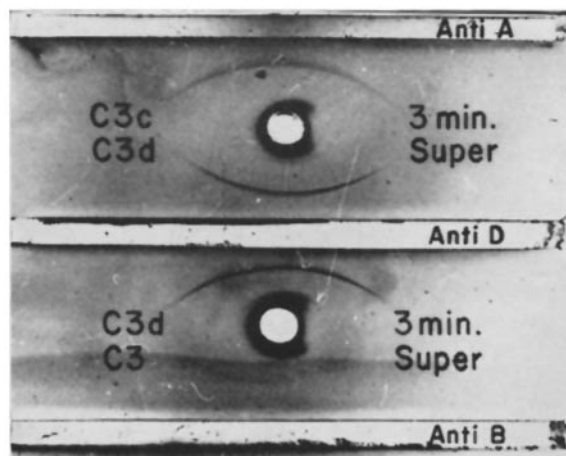


FIG. 2. Agar immunoelectrophoretic analysis of human serum which has reacted for 3 min at 37°C with an immune precipitate.

of molecules, one with a faster mobility in addition to one of the same mobility as C3d. This difference, though small, has been consistent in all experiments. On the other hand, C3c is similar in electrophoretic mobility to  $\beta 1A$  in aged normal human serum (Fig. 5).

Based on criteria previously established (4), C3i, the fluid-phase conversion product of the reaction of purified C3 with C4, 2, has not been identified as a product of the reaction of an immune precipitate with fresh normal human serum. If C3i were present as a prominent breakdown product, since it is a

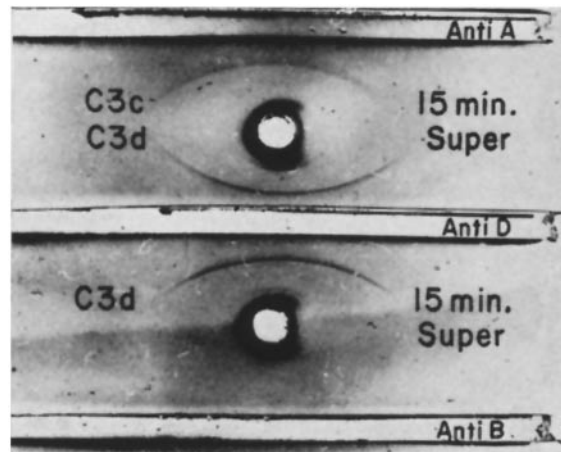


FIG. 3. Agar immunoelectrophoretic analysis of human serum which has reacted for 15 min at 37°C with an immune precipitate.

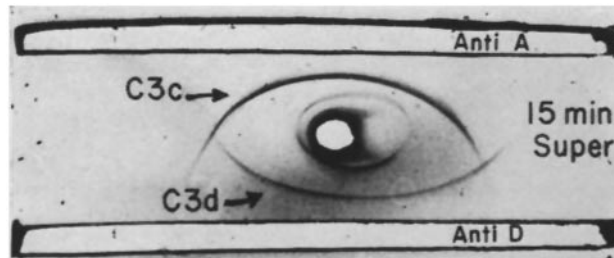


FIG. 4. Agar immunoelectrophoretic analysis of human serum which has reacted for 15 min at 37°C with an immune precipitate. The additional arcs are due to proteins which have precipitated with small amounts of contaminating antibody in the antisera to C3. These arcs have been rendered visible because diffusion has proceeded for 48 hr.

single protein containing both A and D antigens (4), there would be no spurting of precipitin bands made separately with antisera to the A and D antigens as shown in Fig. 4 for C3c and C3d.

*Characteristics of C3 Fluid-Phase Conversion Products in Acrylamide Gel.*—Whereas immunoelectrophoretic analysis in agar of serum reacted with an immune precipitate showed that only two breakdown products are liberated in the fluid phase, acrylamide immunoelectrophoretic analysis revealed one

other conversion product. After the first 30 sec of reaction, there appeared in the fluid phase a protein which was antigenically intact but which had a slightly faster mobility than native C3 (Fig. 6). This protein was relatively stable and persisted in the fluid phase for as long as 1 hr at 37°C. Because it was similar to native C3, reacted with antibody to three antigens, A, B, and D, and did not appear to be a cleavage product, it was given the designation C3x. As will be reported in a subsequent communication, a protein with the same characteristics as C3x in the fluid phase may also be eluted from the immune precipitate.

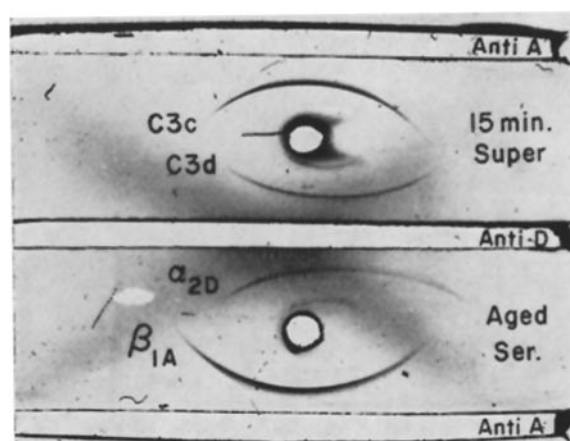


FIG. 5. Comparison of the agar immunoelectrophoretic patterns of serum which has reacted for 15 min at 37°C with an immune precipitate (above) and aged serum (below).

Acrylamide immunoelectrophoretic analysis of the fluid phase delineated two other important features of the C3 conversion products (Fig. 7). As in agar, the product bearing the D antigen (C3d) had a slower mobility than did  $\alpha_2D$  in aged serum. In contrast, however, while the mobilities of C3c and  $\beta_{1A}$  were similar in agar, in polyacrylamide gel the conversion product bearing the A antigen (C3c) had a slower mobility than did  $\beta_{1A}$  in aged serum. Again no protein corresponding to C3i was identifiable in the fluid phase.

*Characteristics of C3i.*—C3i, formed by the reaction of purified C3 and EAC<sub>1,4,2</sub>, reacts with antibody to the A and D antigens of native C3 but, as noted in a previous publication (4), not with antibody to the B antigenic determinant. Observations made in the present study indicating the absence of the B determinant are listed below: (a) C3i reacts only with antisera to A and D antigens by immunoelectrophoretic analysis in agar. (b) A band corresponding to C3i can be developed on an acrylamide gel only by the use of antisera to A and D antigens and not by antisera to the B determinant. (c) On



agar immunoelectrophoretic analysis of a mixture of C3 and C3i, the arc representing C3 may be seen to spur through that for C3i when using a combined anti-A, B, and D antiserum. This is due to the fact that C3 reacts with antibody to the B antigen while C3i does not. There is no spurring when antisera



FIG. 6

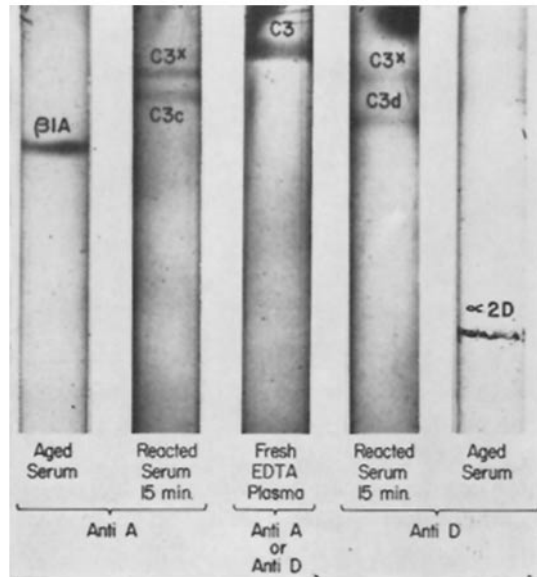


FIG. 7

FIG. 6. Comparison of acrylamide immunoelectrophoretic patterns illustrating C3 in EDTA serum and C3 and C3x in a serum which had reacted with an immune precipitate for 1 min. These gels have been developed in antibody to the A antigen of C3. C3 and C3x would also be visualized in the same pattern with antibody to the B or D antigens of C3. C3c is not visualized in the same pattern with antibody to the A antigen had to be diluted several-fold in order to clearly illustrate C3x. This was necessary because C3x is present in much smaller amounts than C3c at this time interval.

FIG. 7. Acrylamide immunoelectrophoretic patterns of fluid-phase C3 conversion products. The gels to the left of center have been developed with antibody to the A antigen; those to the right with antibody to the D antigen. The center gel contains unreacted EDTA plasma; on either side of the center gel are gels containing serum reacted for 15 min with an immune precipitate; the outer gels contain aged serum. When antibody to the B antigen is used to develop these gels, only C3 and C3x are visualized.

to only A and D antigens are used. (d) When all of the material reacting with anti-B is absorbed from a mixture of purified C3 and C3i by monospecific antiserum, agar immunoelectrophoretic analysis of the resultant solution reveals a protein with the mobility of C3i which reacts only with antibody to A and D antigens.

C3i has a similar electrophoretic mobility in agar to that of C3x. In acrylamide gel the mobilities of C3i and C3x also are very nearly the same. In numer-

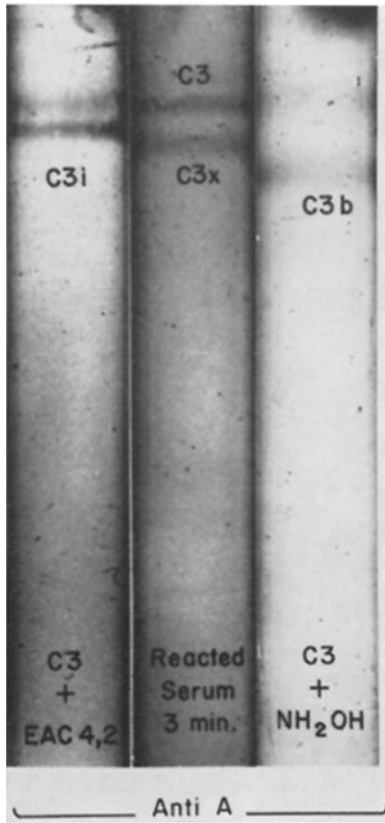


FIG. 8

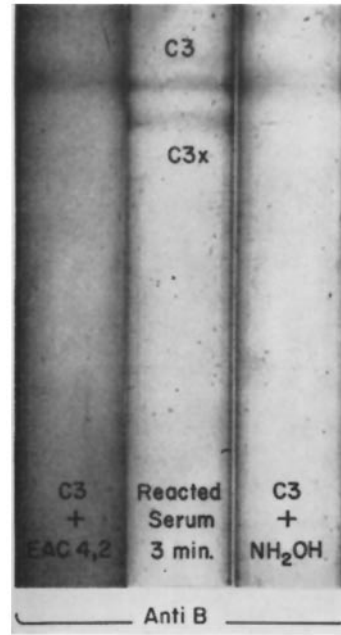


FIG. 9

FIG. 8. Acrylamide immunoelectrophoretic analysis of C3, C3i, C3x, and C3b. The gel to the left contains the fluid phase of the reaction of purified C3 and EAC4, 2. The gel in the center contains serum which has reacted for 3 min with an immune precipitate. The gel on the right contains the end product of the reaction of euglobulin and hydroxylamine. All gels have been soaked in antibody to the A antigen. C3c is not visualized in the center gel for the reason enumerated in the legend of Fig. 6.

FIG. 9. Acrylamide immunoelectrophoretic analysis of the same samples as in Fig. 8. However, the gels have been developed in antibody to the B antigen. Only native C3 and C3x visualize, since C3i and C3b do not consume antibody to the B antigen.

ous determinations, however, there was a consistent and reproducible difference, C3x always having a slightly faster mobility than C3i (Fig. 8). Nevertheless, the major point of difference between the two proteins rests with the fact that C3i does not consume antibody to the B antigen, which C3x does (Fig. 9).

*Characteristics of C3b.*—C3b was produced in the fluid phase by the reaction of purified C3 with trypsin (3) or hydroxylamine (16), or by the acidification of C3i (17). In all of the above situations, C3b, like C3i, consumed antibody to the A and D antigens of native C3 but not to the B antigenic determinant. This has been inferred from the following: (a) C3b reacts only with antisera to the A and D antigens by immunoelectrophoretic analysis in agar. (b) A band corresponding to C3b can be developed on an acrylamide gel only by the use of antisera to A and D antigens and not by antisera to the B determinant.

TABLE I  
*Comparison of C3 Conversion Products*

Product	Demonstrable antigens	Mol wt
C3	A, B, D	235,000 (3)
C3x	A, B, D	?
C3i	A, D	?
C3a	B	8,700 (3)
C3b	A, D	223,000 (3)
C3c	A	151,000 (3)
C3d	D	27,000 (3)
$\beta$ 1A	A	150,000 (25)
$\alpha$ 2D	D	70,000 (25)

(c) When C3b is quantitated for the A, B, and D determinants by the immunoelectrophoretic precipitin method, consumption of antibody to the A and D antigens can be demonstrated; there is no consumption of antibody to the B antigen.

C3b and C3i not only seem to have the same antigenic configurations, but also identical electrophoretic mobilities in agar. By analysis in polyacrylamide gel, however, C3b has a faster mobility than C3i. In addition, C3b also has a faster mobility in acrylamide gel than does C3x.

The major differences in C3x, C3i, and C3b are illustrated in Figs. 8 and 9. Fig. 8 illustrates the relative polyacrylamide gel mobilities of the three proteins. Fig. 9 shows that C3x differs from C3i and C3b by consuming antibody to the B antigen. A summary of some of the characteristics of the C3 conversion products may be found in Table I and Fig. 10.

*Characteristics of C3a.*—C3a, whether produced by the reaction of trypsin (3) and purified C3 or hydroxylamine (16) and purified C3, has a characteristic mobility on acid polyacrylamide gel. As illustrated in Fig. 11 and described

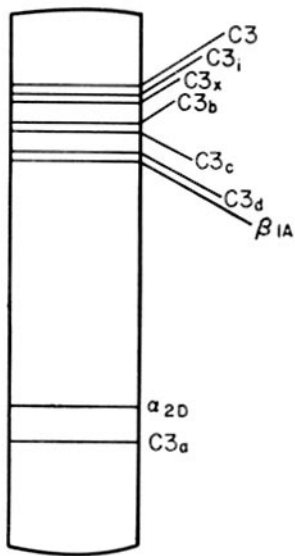


FIG. 10

FIG. 10. Schematic drawing illustrating the relative mobilities of C3 and its conversion products on polyacrylamide gel.

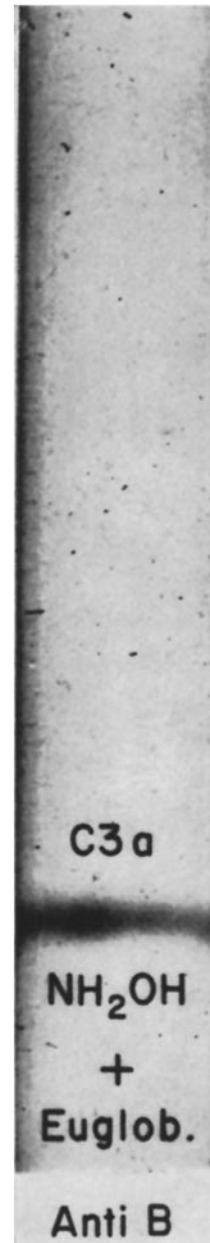


FIG. 11

FIG. 11. Acrylamide immunoelectrophoretic analysis of C3a. The C3a was produced by the reaction of hydroxylamine and euglobulin. The gel has been developed in antibody to the B antigen of C3.

previously by Bokisch et al. (3), it has a marked cathodal migration. C3a contains only the B antigen as demonstrated by the following: (a) A band corresponding to C3a may be developed on an acid gel only by antisera to the B antigen. (b) C3a consumes antibody only to the B antigen and not to A and D antigens. Similar results have been obtained using highly purified C3a derived from trypsin-reacted C3 kindly provided by Dr. H. J. Müller-Eberhard.

*Reaction of Trypsin with Normal Human Serum.*—When trypsin is allowed to react with normal human serum at 20°C for 60 sec (3), the product, subjected to agar immunoelectrophoretic analysis, reacts with the A and D antigens to produce arcs resembling those produced by C3c and C3d (Fig. 12). On

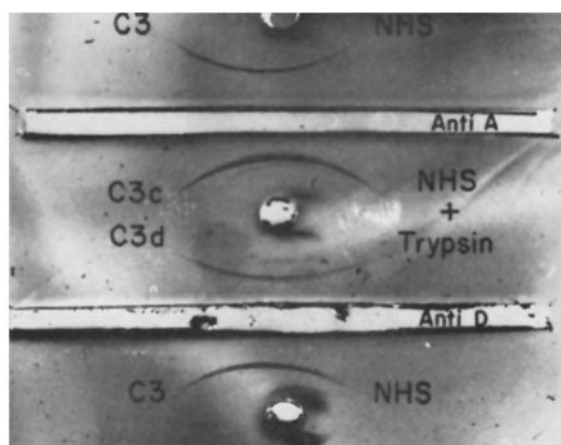


FIG. 12. Agar immunoelectrophoretic analysis of trypsin-treated normal human serum at 20°C for 60 sec. The original serum is shown above.

acrylamide immunoelectrophoretic analysis, however, the trypsin-produced C3c and C3d have slightly faster mobilities than those products obtained when fresh normal human serum reacts with C4, 2 on an immune precipitate. C3b was not found, even under these mild conditions, in contrast to the study by Bokisch et al. (3). If the incubation is continued for 1 hr at 37°C,  $\beta$ 1A and  $\alpha$ 2D are generated, as well as C3c and C3d. After 4 hr at 37°C, all of the A antigen may be found on  $\beta$ 1A and all of the D antigen on  $\alpha$ 2D (Figs. 13, 14).

*Fluid-Phase Kinetics of the B Antigen of Native C3.*—As indicated in previous sections, the B antigenic determinant is demonstrable on native C3 as well as on two conversion products, C3x and C3a. Quantitatively, however, as fresh normal human serum reacts with an immune precipitate at 37°C, the consumption of antibody by the B antigen decreases as native C3 is converted to C3a and C3x. This decrease in antibody consumption is, in large part, due to the fact that the B antigen of C3a is unavailable for combination with antibody

and that C3x has fewer B determinant groups than C3. The kinetics of this reduction of antibody consumption are illustrated in Fig. 15 and are a measure of the rate of the conversion of native C3. A control curve illustrating the

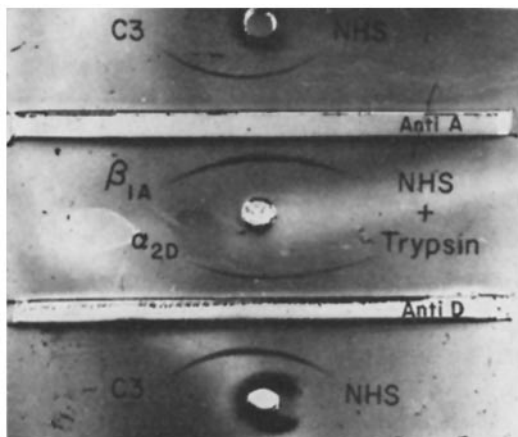


FIG. 13. Agar immunoelectrophoretic analysis of trypsin-treated normal human serum at 37°C for 4 hr is shown in the center panel; the original serum is shown above and below.

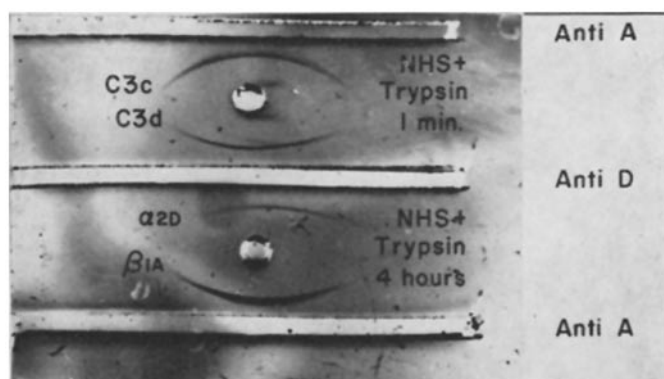


FIG. 14. Agar immunoelectrophoretic analysis of trypsin-treated normal human serum at 20°C for 60 sec is shown above. Serum which has reacted with trypsin for 4 hr at 37°C is shown below.

alterations in the B antigen of C3 as EDTA normal human serum reacts with an immune precipitate is included in Fig. 15. In the presence of EDTA there is no possibility for the formation or binding of C3 convertase and consequently no change in the B antigen or conversion of C3. It is evident that, under conditions where C3 convertase is able to form, consumption of antibody to the

B antigen decreases rapidly, the reduction amounting to almost 50% in the first 60 sec.

These same data are illustrated in Fig. 16 but are expressed as the per cent

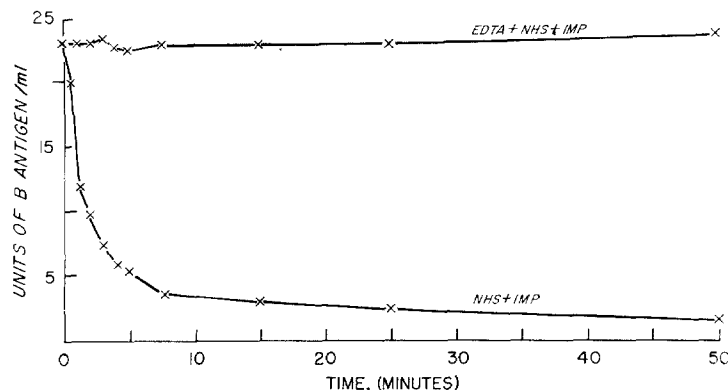


FIG. 15. Kinetics of the B antigen in the fluid phase as normal human serum reacts with an immune precipitate at 37°C. A control curve with EDTA present in a final concentration of 0.02 M serves as a control. Initial C3 concentration is 23  $\mu$  B antigen/ml or 1.2 mg/ml (as  $\beta$ 1A).

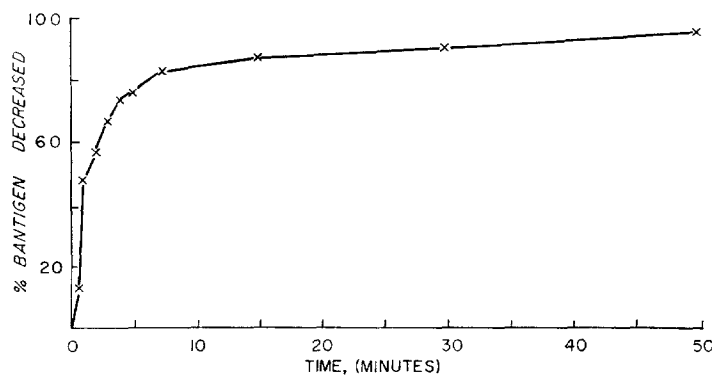


FIG. 16. Per cent of initial C3 converted during the reaction of normal human and an immune precipitate.

of initial B antigen or C3 input that is converted. It is to be noted that the work with acrylamide gel electrophoresis indicates that the B antigen which will consume antibody at any given time in the fluid phase is a summation of the B antigen on unreacted C3 as well as that contained on C3x. It has not yet been possible to quantitate these two proteins separately so as to obtain a more precise determination of the conversion of the native molecule.

*Kinetics of the Fluid-Phase A and D Antigens of C3.*—As serum reacts with an immune precipitate, the A antigen originally present on native C3 may be found on three different conversion products, C3x, C3b, and C3c. C3x is present in the fluid phase as well as bound to the immune precipitate; C3b is only present in the bound phase, while C3c is only found in the fluid phase. Similarly, the D antigen, originally present on native C3, may be found on C3x, C3b, and C3d. C3d, like C3c, is only present in the fluid phase. The fluid-phase measurements of the total A and D antigens at any given time, therefore, reflect the kinetic changes in each of these proteins. The results of

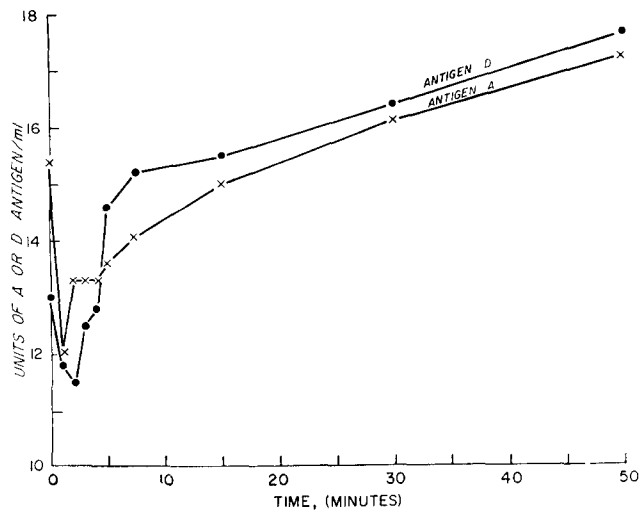


FIG. 17. Kinetics of the A and D antigens in the fluid phase as normal human serum reacts with an immune precipitate at 37°C.

such measurements may be seen in Fig. 17. It is clear that in the first few minutes of reaction the values for both A and D antigens in the fluid phase decrease in amounts corresponding to 20% for the A and 10% for the D antigen. This decrease corresponds to the deposition of C3 products on the immune precipitate. Despite the fact that deposition continues for nearly 5 min (18, 19),<sup>4</sup> however, the amounts of fluid-phase A and D antigens do not continue to decrease. This reflects the fact that as C3 is activated, only a small percentage of the activated molecules are deposited, the remainder cleaving in the fluid phase. As cleavage occurs, the number of antigenic sites increases but to a lesser extent than when serum ages (12). This increase accounts for the rise in

<sup>4</sup> Spitzer, R. E., and A. E. Stitzel. The antigenic and molecular alterations of the third component of human complement during an immune reaction. Demonstration of two deposition products from normal human serum. Manuscript in preparation.



value for fluid-phase A and D antigens before deposition ceases at about 5 min. After 5 min of reaction, the increase in the amount of A and D antigens is more than would be expected from cleavage of native C3 and presumably reflects the removal of bound C3 by serum factors and its subsequent cleavage in the fluid phase (18, 19).<sup>4</sup>

*Aging of C3 and C3x.*—Normal human serum which had reacted with an immune precipitate for 3 min was separated by centrifugation and allowed to age for 7–14 days at 37°C. The B antigen concentration at the onset of aging was 15 units/ml. From previous work (12), it would be expected that with aging, if that protein containing the B antigen in the fluid phase were truly native unreacted C3, there would be loss of the B antigen, an increase in

TABLE II  
*Effect on Antibody Consumption to Determinants of C3 with Aging of Serum Previously Reacted with an Immune Precipitate at 37°C for 3 Min*

	Before aging	After aging	Expected after aging	Actual increase
	<i>units/ml</i>	<i>units/ml</i>	<i>units/ml</i>	<i>% of expected*</i>
B antigen	15‡	0.0	0.0	—
A antigen	19.2	23.5	32.7	31
D antigen	19.5	17.6	42.0	0

\* The expected number of units after aging was calculated by ascertaining the units of A or D antigen as C3 at the onset of aging and multiplying by factors expressing the amount of expansion that is obtained with the same serum when it is aged without prior reaction with an immune precipitate (12).

‡ The serum used for the experiment had an unusually high C3 concentration; for this reason, the concentration of B antigen after 3 min of reaction is higher than that shown in other experiments (Figs. 16, 17).

antibody consumption to the A and D antigens, and the resultant formation of  $\beta$ 1A and  $\alpha$ 2D. In 15 separate experiments, however, all supernatants upon aging showed little or no change in antibody consumption by the A and D antigens as the B antigen disappeared (Table II). Acrylamide immunoelectrophoretic analysis of the aged supernatant identified the A antigen-containing protein as  $\beta$ 1A and the D antigen-bearing product as C3d. No C3c or  $\alpha$ 2D were present. Thus, it appears that the native C3 molecules have been altered by brief contact with an immune precipitate, so that upon aging they fail to expose additional antigenic sites. In addition, this alteration prevents cleavage of the molecule in the usual fashion so that the products obtained upon aging are  $\beta$ 1A and C3d. It is noteworthy that the C3x present in the supernatant before aging also disappeared. It must therefore be assumed that C3x, on aging, also does not expose additional antigenic sites.

Since the reacted serum before aging contained a mixture of “altered” C3,

C3x, C3c, and C3d, while after the aging only  $\beta$ 1A and C3d could be identified, the following conclusions can also be inferred: (a) Altered C3 and C3x must convert to  $\beta$ 1A and C3d upon aging. (b) C3c must convert to  $\beta$ 1A upon aging. (c) C3d is not altered by the aging process.

*Heat Stability of C3 and C3x.*—Normal human serum which had reacted with an immune precipitate for either 30 sec or 3 min at 37°C, was centrifuged and then heated to 56°C for 60 min. The B antigen present in the reacted serum completely disappeared and C3c and C3d were produced. In contrast, if unreacted normal human serum is heated to 56°C for 60 min, the B antigen is reduced by only 30% and a small amount of C3c and C3d is generated. These data are illustrated in Table III and suggest that the heat stability of native C3 in human serum has been grossly altered by a brief contact with an immune precipitate.

TABLE III  
*Effect of Heating on B Antigen of C3 in Normal Serum and in a Serum Reacted with an Immune Precipitate*

	B antigen before heating	B antigen after heating*	Decrease
	<i>units/ml</i>	<i>units/ml</i>	<i>%</i>
Normal human serum	30	21	30
30 sec reacted serum‡	18	<0.5	100
3 min reacted serum‡	7.5	<0.5	100

\* 56°C for 60 min.

‡ Serum previously reacted with an immune precipitate at 37°C for either 30 sec or 3 min.

#### DISCUSSION

The use of monospecific antisera to each of the three major antigenic determinants of C3 provides an investigative tool which allows identification of the products of the in vitro conversion of serum C3 without interference from other serum proteins. Breakdown products produced in a variety of ways may be identified by their reaction with specific antibodies after electrophoresis in agar or acrylamide gel. The present study has dealt mainly with the products of C3 found in the fluid phase when the C3 in normal human serum ages or reacts with C3 convertase or trypsin. Of interest are the differences in the breakdown products produced in serum as compared with those found in the reaction with purified complement components. While the use of the C3 antigenic determinants affords a sensitive means to define these products, certain deficiencies still exist, the resolution of which must await better analytical techniques.

C3, whether in purified form or in normal human serum, can be defined as a protein bearing three antigenic determinants, A, B, and D, with distinctive

electrophoretic mobilities in agar and acrylamide gel. When purified C3 is reacted with isolated  $\overline{C4,2}$ , C3 is converted to C3i which reacts only with the A and D antigens (4) and exists only in the fluid phase (20). In addition to the apparent absence of the B antigen, C3i differs from native C3 by having a faster electrophoretic mobility in agar, a greater number of available sulfhydryl bonds, and an  $s_{20}$  rate of 9.0 as compared to 9.5 for C3 (1, 20, 21). Reasoning from this data, it would be expected that the formation of C3i from C3 would entail the loss of a small fragment. None, however, has been demonstrated (1, 20, 21). Thus, it is necessary to assume a change in conformation with a loose complex of C3a and C3b, rather than complete cleavage of C3 as C3i is formed. Further evidence to support this view is the fact that when C3i is acidified to pH 3, a small basic fragment known as C3a dissociates (17, 22). C3a has been shown to bear at least one antigenic determinant in common with native C3 (3), which in the present study has been documented as the B determinant. It is clear that the B antigen must also be present on C3i but unavailable for combination with antibody by virtue of the configurational change in the molecule.

The portion of the C3i molecule remaining after dissociation of C3a in acid is known as C3b (20). Despite the loss of C3a which has an approximate weight of 10,000 (3), the  $s_{20}$  rate of C3b is essentially the same as C3i (3). It also consumes antibody to the same antigenic determinants, A and D, and has the same electrophoretic mobility in agar as C3i. By electrophoretic analysis in acrylamide gel, however, C3b may be shown to be distinct from C3i. C3b may also be found on an erythrocyte, after the reaction of C3 and  $\overline{EAC1,4,2}$  at neutral pH (20), and is the only form of bound C3 which may be obtained in pure systems (23). The C3b under these conditions appears to be identical with the C3b formed from C3i at low pH (20).

The present study indicates that when human serum is used as a source of complement, the reaction of C3 with  $\overline{C4,2}$  produces somewhat different conversion products. Foremost, C3i is not identifiable in the fluid phase. Whether it is simply never produced or is rapidly degraded by serum factors, as intimated by Müller-Eberhard (24), is not known. Not only is C3i not identifiable, but, in addition, its breakdown products, C3a and C3b, have not been found in the fluid phase. Furthermore, C3a functional activity, i.e. anaphylatoxin activity, has been reported to be minimal or absent when the complement system in human serum is activated (3). This inability to demonstrate C3a or C3 anaphylatoxin activity in serum may be due to a number of factors. C3i, if it is formed, might be degraded in such a fashion that C3a is simply not generated; alternatively and more likely, if C3i is cleaved so that C3a is formed, the C3a may be rapidly inactivated by the C3a inactivator present in normal human serum (3). In an effort to demonstrate C3a, the pH of a reacted serum was lowered to 3.2. If C3a were generated, the increased  $H_3O^+$  concentration would

facilitate its liberation and at the same time block the action of the C3a inactivator, as described by Bokisch et al. (3). Unfortunately, below pH 5 the B antigenic determinant is rendered partially unreactive so its presence cannot be clearly demonstrated by this maneuver.

As will be shown in subsequent publications (19),<sup>4</sup> C3b, while absent from the fluid phase, is, nonetheless, found in the solid phase. It may be eluted by EDTA Veronal-buffered saline from an immune precipitate, and from this source shows the same antigenic configuration and electrophoretic mobility in agar as C3i formed in pure systems. C3b may be differentiated from C3i by its mobility on polyacrylamide gel.

In the present study, a new conversion product which is not C3i, C3a, or C3b has been found in the fluid phase, as well as on the immune precipitate (18, 19).<sup>4</sup> This conversion product has been designated C3x. C3x, like C3, bears all three antigenic determinants but differs from it in that C3x has a slightly faster mobility in both agar and polyacrylamide gel. It differs from C3i and C3b in its antigenic configuration, in its electrophoretic mobility in acrylamide gel, and in its stability in serum. Additional data to be presented subsequently amply support these differences (19).

C3x has not been found when purified C3 reacts with  $\overline{\text{EAC4,2}}$ . The qualitative data of this paper do not allow assumptions as to the origin of C3x. However, further work (18, 19) strongly suggests that C3x is a product of a serum conversion system unrelated to C3 convertase and has considerable biologic activity.

Finally, two proteins considered to be the counterparts of  $\beta 1A$  and  $\alpha 2D$  in aged serum have been found in the fluid phase. These, by World Health Organization nomenclature (25), are designated C3c and C3d. Whether they are formed directly from native C3, arise from C3x, or are degradation products of unstable fluid phase C3b, is not clear. C3c clearly differs from  $\beta 1A$  and C3d from  $\alpha 2D$ , although they bear exclusively the A and D antigens respectively. By mobility in agar, C3d most resembles the  $\alpha 2D$  found when fresh human serum reacts with hydrazine (4). C3c and C3d are similar but not identical to the trypsin-generated fragments of the same designations. A comparison of the C3 conversion products both in isolated systems and in serum may be found in Table I and Fig. 10.

The products of the reaction of trypsin and normal human serum appear to occupy a middle ground between those produced when  $\overline{\text{C4,2}}$  reacts with C3 in serum and those produced by the aging of serum. At short incubation times, C3c and C3d are liberated; with longer times  $\beta 1A$  and  $\alpha 2D$  are formed. Whether  $\beta 1A$  and  $\alpha 2D$  are further breakdown products of C3c and C3d or direct cleavage products of C3 by trypsin is not known. As noted above, the C3c and C3d generated by trypsin are slightly different in their electrophoretic mobilities in acrylamide from the C3c and C3d generated by  $\overline{\text{C4,2}}$  in serum. These differences

in mobility are not totally unexpected. The data of Bokisch et al. (3), for the differences in C3a produced by trypsin and  $\overline{C4_2}$ , lead one to suspect different cleavage points in a relatively select area of the C3 molecule. The observation that the C3c and C3d produced are also slightly different supports this view.

The kinetic data for B antigen disappearance indicate that C3 breakdown occurs very rapidly upon addition of an immune precipitate to fresh human serum. Judging by the loss of the B antigen, 50% of the C3 is converted in 1 min and over 90% by 60 min. It is to be emphasized that these are at best only minimum values since a portion of the B antigen quantitated in the fluid phase is present as C3x, which in itself can be considered a conversion product. Moreover, acrylamide gel studies of the fluid phase after 15 min of reaction with an immune precipitate indicate that most, if not all, of the B antigen-bearing protein is C3x. Hence, it appears that native C3 is converted to another form very rapidly after the immune precipitate addition.

Of greater interest is the fact that even before it is converted to C3x, the C3 molecule appears to be altered by the presence of the precipitate. There are two observations which reveal this alteration. First, within 3 min after addition of the immune precipitate to human serum, the native C3 remaining unconverted is changed by the precipitate so that it does not age in vitro in the same manner as does C3 which has not contacted a precipitate. Previous reports (12) have indicated that when serum containing native C3 is allowed to age in vitro, the formation of  $\beta 1A$  and  $\alpha 2D$  result in the consumption of more antibody to A and D antigens than did the native C3. Thus, with aging, antibody consumption to the A antigen increases by a factor of 1.6–1.8 and that to the D antigen by 2.5–3.0. It would be predicted from this data that serum which has reacted with an immune precipitate for a short time and still contains native unreacted C3, would, on aging, demonstrate an increased consumption of antibody to A and D determinants as  $\beta 1A$  and  $\alpha 2D$  were formed. This, however, was not observed. Antibody consumption to the A and D antigens as shown in Table II did not increase, or increased much less than would be predicted from experience with aged serum. Further, with aging, the ultimate breakdown products are  $\beta 1A$  and C3d rather than  $\beta 1A$  and  $\alpha 2D$ . This observation indicates that the course and characteristics of C3 breakdown produced by aging are materially modified by the addition for a 3-min period of an immune precipitate to the serum. The second point of evidence indicating a primary change in C3 is provided by the comparison of the heat stability of native C3 and the C3 remaining after reaction of normal serum with an immune precipitate for as short a time as 30 sec. When human serum so treated is heated to 56°C for 1 hr, the B antigen of C3 is completely destroyed. In contrast, when unreacted serum is heated to 56°C for 1 hr there is minimal reduction in the B antigen of C3. These observations would indicate that the C3 molecule, even before conversion by an immune precipitate to C3x, C3c, or C3d, very

quickly undergoes a distinct alteration when in contact with an immune precipitate manifested by a change in its reactivity with respect to aging and with respect to its heat lability.

Finally, although the fluid-phase kinetics of the A and D antigens represent a complex situation, their consideration reveals an important concept. The data of Müller-Eberhard et al. (1), for the reaction of purified C3 with isolated C4<sub>2</sub>, indicated that the ratio of converted C3 to bound C3 was relatively constant throughout the reaction period. If the same were true when serum reacts with an immune precipitate, one would expect that as long as deposition were proceeding the amount of A or D antigen in the fluid phase would either slowly decrease in a manner analogous to the B antigen or increase if the unfolding of new antigenic sites during cleavage were very great. However, as illustrated in Fig. 17, there is a diphasic curve in the first 4 min when deposition is occurring, implying that in serum the ratio of converted to bound C3 is not constant. Presumably, this lack of constancy is the result of the fact that C3 is converted and bound both as C3x and C3b (18, 19).<sup>4</sup>

It is quite clear that the conversion products of C3 resulting from the reaction of an immune precipitate with normal human serum are more complex than previously thought. By the same token it is equally clear that the reactions observed using purified complement components may not always be indicative of those that occur in the milieu of serum. The differences appear to be more than minor. As one example, C3x seems to represent a major conversion product of C3 that is not generated with purified complement components but is present in serum. Furthermore, the kinetics of these reactions in serum, which will be enlarged upon in subsequent communications, are considerably different from those in isolated systems. If the role of the complement system in pathologic studies is to be characterized, it would seem appropriate to explore the reaction in a milieu more closely approximating *in vivo* conditions. Further studies on these molecular and antigenic alterations of C3 in serum, as well as the functional capabilities of its conversion products, are now in progress.

#### SUMMARY

During the reaction of an immune precipitate with fresh human serum, C3 undergoes a number of molecular alterations with the formation of conversion products differing from those obtained when purified components react. Those products which remain in the fluid phase, the subject of the present paper, have been identified by their reaction with monospecific antisera to the three antigenic determinants of C3, A, B, and D, after electrophoresis in agar or polyacrylamide gel.

When purified C3 reacts with EAC<sub>1,4,2</sub>, C3i is found in the fluid phase. C3i, a loose complex of C3a and C3b, is in a conformational state whereby only the A and D antigens, present on its C3b portion, will consume antibody. The B

antigen, present on the C3a portion of C3i, is unavailable for combination with antibody until C3i dissociates.

In the fluid phase of the reaction of an immune precipitate with whole serum, C3i, C3a, and C3b, formed when purified components react, cannot be found. Instead the end products of the reaction appear to be C3c, which contains the A antigen, and C3d, which contains the D antigen. C3c and C3d are similar to the  $\beta$ 1A and  $\alpha$ 2D produced by the aging of serum but differ in their mobilities in acrylamide gel and in agar. The C3c and C3d generated by an immune precipitate also differ slightly from the C3c and C3d produced by the reaction of trypsin with C3 in whole human serum.

As human serum reacts with an immune complex, native C3 appears to undergo a primary alteration before conversion. This alteration results in a molecular species of C3 which is labile at 56°C for 30 min, fails to expose additional A and D antigenic sites upon aging, and which forms  $\beta$ 1A and C3d rather than  $\beta$ 1A and  $\alpha$ 2D during aging.

In addition to this altered form of native C3, a new conversion product, C3x, is formed as whole serum reacts with an immune complex. C3x is not found in systems utilizing pure complement components. C3x is like C3 in that it bears all three antigenic determinants but differs in that it has a slightly faster mobility in polyacrylamide gel than does native C3. C3x is not only found in the fluid phase but is also bound to the immune precipitate.

Finally, the fluid-phase kinetics of each of the antigens of C3 have been determined as normal human serum reacts with an immune precipitate. These illustrate that nearly the entire population of native C3 molecules undergoes conversion rapidly as manifested by the disappearance of the B antigen from the fluid phase. Moreover, the kinetics of the fluid-phase A and D antigens reflect that the conversion of C3 in serum is quantitatively not the same as when purified C3 reacts with C4<sub>2</sub>.

The authors wish to acknowledge the able technical assistance of Mrs. Mary Wang.

#### REFERENCES

1. Müller-Eberhard, H. J., A. P. Dalmaso, and M. A. Calcott. 1966. The reaction mechanism of  $\beta$ 1C-globulin (C3) in immune hemolysis. *J. Exp. Med.* **123**:33.
2. Müller-Eberhard, H. J., and U. Nilsson. 1960. Relation of a B<sub>1</sub>-glycoprotein of human serum to the complement system. *J. Exp. Med.* **111**:217.
3. Bokisch, V. A., H. J. Müller-Eberhard, and C. G. Cochrane. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* **129**:1109.
4. West, C. D., N. C. Davis, J. Forristal, J. Herbst, and R. Spitzer. 1966. Antigenic determinants of  $\beta$ 1C-globulin. *J. Immunol.* **96**:650.
5. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of  $\beta$ 1F-globulin from

- human serum and its characterization as the fifth component of complement. *J. Exp. Med.* **122**:277.
6. Scheidegger, J. J. 1955. Une micro-methode de l'immuno-electrophorese. *Int. Arch. Allergy Appl. Immunol.* **7**:103.
  7. West, C. D., V. Hinrichs, and N. H. Hinkle. 1961. Quantitative determination of the serum globulins  $\beta$ 2A and  $\beta$ 2M by immunoelectrophoretic analysis. *J. Lab. Clin. Med.* **58**:137.
  8. Wright, S. T. 1959. A quantitative serum agar technique. *Nature (London)*. **183**:1282.
  9. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404.
  10. Müller-Eberhard, H. J., U. Nilsson, and T. Aronson. 1960. Isolation and characterization of two  $\beta$ 1-glycoproteins of human serum. *J. Exp. Med.* **111**:201.
  11. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (London)*. **195**:281.
  12. West, C. D., S. Winter, J. Forristal, and N. C. Davis. 1968. Effect of aging of serum on consumption of antibody by  $\beta$ 1C-globulin determinants; evidence for circulating breakdown products in glomerulonephritis. *Clin. Exp. Immunol.* **3**:1.
  13. Gigli, I., and R. A. Nelson. 1968. Complement dependent immune phagocytosis. *Exp. Cell Res.* **51**:45.
  14. Nelson, R. A., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of the nine components of hemolytic complement in guinea pig serum. *Immunochemistry*. **3**:111.
  15. Borsos, T., and H. J. Rapp. 1967. Immune hemolysis: A simplified method for the preparation of EAC'4 with guinea pig or with human complement. *J. Immunol.* **99**:263.
  16. Budzko, D. B., and H. J. Müller-Eberhard. 1969. Anaphylatoxin release from the third component of human complement by hydroxylamine. *Science (Washington)*. **165**:506.
  17. Müller-Eberhard, H. J., M. J. Polley, and M. A. Calcott. 1967. Formation and functional significance of a molecular complex derived from the second and fourth component of human complement. *J. Exp. Med.* **125**:359.
  18. Spitzer, R. E., A. E. Stitzel, V. L. Pauling, C. D. West, and N. C. Davis. 1970. Binding of C3 on an immune complex: Evidence for two distinct deposition products. *Fed. Proc.* **29**:304.
  19. Spitzer, R. E., A. E. Stitzel, V. L. Pauling, N. C. Davis, and C. D. West. 1971. The activation of C3 by antibody and a non-convertase dependent mechanism in normal human serum: generation of a new C3 conversion product with unique biologic activity. *Int. Congr. Microbiol. Proc.* In Press.
  20. Müller-Eberhard, H. J. 1968. The serum complement system. Textbook of Immunopathology. P. A. Miescher and H. J. Müller-Eberhard, editors. Grune and Stratton Inc., New York. **1**:33.
  21. Müller-Eberhard, H. J. 1969. Complement. *Annu. Rev. Biochem.* **38**:389.
  22. Dias, da Silva, W., J. W. Eisele, and I. H. Lepow. 1967. Complement as a mediator of inflammation. III. Purification of the activity with anaphylatoxin properties



- generated by interaction of the first four components of complement and its identification as a cleavage product of C3. *J. Exp. Med.* **126**:1027.
23. Dalmasso, A. P., and H. J. Müller-Eberhard. 1967. Physico-chemical characteristics of the third and fourth components of complement after dissociation from complement cell complexes. *Immunology.* **13**:293.
  24. Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement, *Advanc. Immunol.* **8**:1.
  25. Nomenclature of complement. 1968. *Bull. W. H. O.* **39**:935.