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STUDIES ON THE THIRD COMPONENT (C'3) OF GUINEA PIG COMPLEMENT[1,2]

I. PURIFICATION AND CHARACTERIZATION

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SUMMARY The third component of guinea pig complement (C'3) was isolated and purified by four serial column chromatographies of modified midpiece fraction on Sephadex G-200, carboxymethyl (CM) and diethylaminoethyl (DEAE) cellulose and then Sephadex G-200.

The purified C'3 preparation formed a single zone in the β_1 globulin region on disc electrophoresis. On immunodiffusion analyses, it gave a single precipitation line against either anti-guinea pig whole serum antiserum or anti-purified C'3 antiserum. The latter antiserum prepared by immunizing rabbits with the purified C'3 preparation showed a single precipitation line against fresh guinea pig whole serum. When the immunoelectrophoretic plate was covered with an agar layer containing EAC'1a,4 cells and C'2, C'5, C'6, C'7, C'8 and C'9, a hemolytic zone clearly bordered by the precipitation arc appeared in the β_1 globulin region.

Rabbit anti-purified C'3 antiserum inhibited both the hemolytic and immune adherence (IA) reactivities and agglutinated EAC'1a, 4, 2a, 3 cells.

C'3 showed higher hemolytic reactivity in medium of low ionic strength. Methods of titration under various conditions were compared and discussed.

INTRODUCTION

Classical third component of complement has now been resolved into 6 components, i.e., C'3, C'5, C'6, C'7, C'8 and C'9. C'3, designated by the new nomenclature (NELSON, 1967), was discovered as a third component of them by RAPP (MAYER, 1961) and named C'3c. C'3c was found to react after C'2 and before C'3b (now known as C'5) in immune hemolysis by NISHIOKA and LINSCOTT (1963). They also demonstrated that C'3c is the essential

[1] The complement components in guinea pig, human and rabbit serum are now designated by numbers following the nomenclature of NELSON (1967). In brief, the new nomenclature represents the sequential reaction of nine components as C'1, C'4, C'2, C'3, C'5, C'6, C'7, C'8, and C'9 which orginally corresponded to C'1, C'4, C'2, C'3c, C'3b, C'3e, C'3f, C'3a and C'3d.

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component of the immune adherence (IA) reaction. MÜLLER-EBERHARD and NILSON (1960), on the other hand, isolated a β_1-glycoprotein from fresh human serum and designated it as β_{1C}-globulin. They demonstrated that it stabilized EAC'1a, 4, 2a cells. It has now been established that β_{1C}-globulin, also designated as C'3 by MÜLLER-EBERHARD, corresponds to guinea pig C'3 (C'3c) (NELSON, 1967), although the original β_{1C}-globulin was known to contain C'3, C'5, C'6 and C'7.

For analysis of the C'3 reaction step, guinea pig C'3 was isolated and purified in this laboratory. A highly reproducible method for purifying guinea pig C'3 and some characteristics of this component are described in this paper.

MATERIALS AND METHODS

1. *Diluents*

Isotonic veronal-buffered saline containing 0.1% gelatin, 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$ (VB-saline) was prepared according to the description of MAYER (1961a). Isotonic veronal-buffered sucrose solution (VB-sucrose) was prepared like VB-saline except that it contained 9.25% (w/v) sucrose instead of NaCl. An isotonic veronal-buffered solution of low ionic strength was made by mixing VB-saline and VB-sucrose in appropriate proportions. A mixture of equal volumes of VB-saline and VB-sucrose (VBSS) was also used. VB-saline containing 0.01 M ethylene-diamine tetraacetate (0.01 M EDTA-VB-saline) was prepared by mixing 1 part of 0.1 M trisodium ethylene-diamine tetraacetate (pH 7.5 to 7.7) with 9 parts of VB-saline prepared without added Mg^{++} or Ca^{++}.

2. *Sheep erythrocytes (E), rabbit hemolysin (A) and guinea pig serum*

These were obtained and standardized as described by MAYER (1961a).

3. *Sensitized erythrocytes (EA) and intermediate cells of immune hemolysis*

These were prepared as described in the previous report (INOUE *et al.*, 1967).

4. *Human erythrocytes for the immune adherence reaction*

Human erythrocytes of group O were obtained from healthy members of this laboratory.

5. *Partially purified C'2 preparations*

These were prepared and titrated by the method of BORSOS (1961).

6. *Separation and isolation of C'5, C'6, C'7, C'8 and C'9*

These components were isolated according to the routine methods outlined in the descriptions of NELSON *et al.* (1966) and INOUE *et al.* (1965, 1966 and 1967). All column chromatographies, except for routine isolation of C'2 (BORSOS *et al.*, 1961), were carried out in a cold room.

7. *Measurement of ionic strength*

The ionic strengths of the diluent or solutions used for chromatography were checked by measuring their conductivity with a Serfass conductivity bridge, model RCM15B1 (A. H. Thomas Co., Philadelphia, Pa.).

8. *Measurement of immune adherence*

For routine work, the immune adherence (IA) reactivity of C'3 was measured by a Microtiter plate method. The test sample was diluted in VB-saline with dilution-loops on a Microtiter plate. One drop of partially purified C'2 diluted with VB-saline, supplying about 50 effective molecules per cell, and one drop of EAC'1a,4 cell suspension at a concentration of 4.0×10^7 cells/ml were delivered into each of the cups by pipette droppers. The plate was incubated at 37°C for 15 to 25 min [depending on the *t max* of EAC'1a,4 for EAC'1a,4,2a generation (BORSOS *et al.*, 1961)] with continuous shaking. One drop of human erythrocytes at a concentration of 1.0×10^8 cells/ml, was then delivered into the cups. The final volume of the reaction mixtures was 5 drops (5×0.025 ml). The plate was vibrated on a mechanical vibrator at 37°C for 15 min. It was then stood at 37°C for 60 min before reading. The titer was expressed as the highest dilution showing a 2+ reaction ($C'IA_{50}$).

The titers of C'3 reactivity of a particular sample obtained by various methods can vary with the different reagents employed. However, the rough ratio of the titers of a given sample obtained by different methods can be estimated as follows; hemolytic titer on a Microtiter plate 1, IA on a Microtiter 2 to 3, hemolytic titer by the tube method about 5, IA in tube 50 to 100.

9. *Disc electrophoresis*

This was carried out in an EC470 vertical gel electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.) using 5% cyanogum 41 in veronal-acetate buffer (pH 8.6, μ=0.05) as a supporting medium at 100 V for 10–12 hr according to the method given in Technical Bulletin 128 of E-C Apparatus Corp. After separation, the fractions were stained by amidoblack 10B and unfixed dye was removed in an EC 489 Electrophoretic Destainer (E-C Apparatus Corp.).

10. *Antisera*

a) Rabbit anti-guinea pig C'3 antiserum

Rabbits received a subcutaneous injection of 3.0 ml of purified C'3 preparation (concentrated by ultrafiltration to give an OD_{280} of about 0.5 and emulisified with an equal volume of complete Freund's adjuvant) into their foot pads and neck, simulataneously with an intravenous injection of 0.5 ml of the concentrated C'3. After an interval of one week, they received an intravenous injection of 0.5 ml of the antigen. On the 5th day after each injection the serum specimens were examined by immuno-diffusion against C'3. The rabbits were bled on the 7 to 10 days after the last immunization. After separation, the serum was diluted with distilled water to give μ=0.015 and adjusted to pH 7.5 with 0.015 N HCl. It was then passed through a column of DEAE cellulose equilibrated with 0.01 M NaCl in 0.005 M tris (hydroxymethyl) aminomethane (Tris) HCl buffer, pH 7.5. The effluent was concentrated to the volume of the original serum by ultrafiltation. These isolated 7S fractions contained no detectable complement components, and were used as rabbit anti-guinea pig C'3 antiserum in this work.

b) Rabbit anti-guinea pig whole serum antiserum

Rabbits were immunized at intervals of 3 to 4 weeks with 3.0 ml of fresh guinea pig whole serum emulsified in an equal volume of complete Freund's adjuvant each time injected into their foot pads and neck. Serum specimens were taken 2 to 3 weeks after each immunization, and checked by immunoelectrophoresis against guinea pig whole serum. The rabbits were bled 2 to 3 weeks after the last immunization. The 7S fractions were isolated similarly to anti-C'3 antiserum and used as rabbit anti-guinea pig whole serum antiserum in this work.

11. *Ouchterlony's double diffusion method*

This was carried out with 2% agarose in isotonic veronal-buffered saline, pH 7.4, μ=0.147, as supporting medium.

12. *Immunoelectrophoresis*

This was carried out according to a modification of the method of SCHEIDEGGER (1955) using 2% agarose in veronal-acetate buffer, pH 8.6, μ=0.05.

13. *Hemolytic reactivity of C'3 on the immunodiffusion plate*

After development of precipitation lines, the plate was covered by melted 0.75% Special Agar Noble (Difco) (kept at 45° to 47°C) containing EAC'1a, 4 at a concentration of 1.0×10^8 cells/ml, partially purified C'2 (about 200 effective molecules per cell) and moderate excesses of C'5, C'6, C'7, C'8 and C'9 in VB-saline. After gelification the plate was incubated at 37°C for 90 min.

14. *Chemicals*

Diethylaminoethyl (DEAE) cellulose was obtained from Brown Co., Berlin, N. H. Carboxymethyl (CM) cellulose (Serva) was from Gallard-Schlesinger Chemical Mfg. Corp., Long Island, N. Y. Sephadex G-200 was obtained from Pharmacia, Uppsala, Sweden. NaCl, Na-5, 5-diethyl barbiturate, 5, 5-diethyl barbituric acid and gelatin were from Merck AG., Darmstadt, Germany. Tris (hydroxymethyl) aminomethane (Tris) and sucrose were obtained from Nakarai Chemicals, Ltd., Kyoto, Japan. Ethylenediamine tetraacetate (EDTA), 2Na and 4Na were obtained from Wako Pure Chemicals, Ltd., Osaka, Japan.

RESULTS

1. *Purification of guinea pig C'3*

Forty ml of guinea pig serum were diluted with 20 ml of ice-cold distilled water in an ice bath, and were adjusted to pH 5.6 by adding cold 0.1N HCl using a magnetic stirrer. After gentle stirring at 0°C for 45 min, the serum was centrifuged in the cold. The precipitate was separated and was used as a source of C'5. The supernatant was dialyzed overnight against about 5 liters of cold distilled water. The serum was then centrifuged in the cold. The

supernatant was removed and used as a source of C'6, C'7, C'8 and C'9. The precipitate was dissolved in 1 ml of 5 times concentrated VB stock solution (MAYER, 1961a), and was diluted with 4.0 ml of cold distilled water. This solution showed $OD_{280}=37.5$ and 6,561 $C'IA_{50}$/ml using the Microtiter IA method. It was then applied on the column of Sephadex G-200 (30×900 mm) equilibrated with isotonic veronal-buffered saline (VB-saline without gelatin, Ca^{++} and Mg^{++}) (pH 7.4, $\mu=0.147$), and was eluted with the same buffer. Fractions of about 12 ml were collected using an automatic fraction collector. As shown in Fig. 1, the peak of C'3 activity was located in 7S globulin fractions and was separated from those of C'9.

Fractions 12 to 16 were pooled and mixed with one ninth volume of 0.2 M acetate buffer, pH 5.0. The pool was adjusted to pH 5.0 with 0.1 N acetic acid, and then diluted with cold 0.02 M acetate buffer, pH 5.0 to give $\mu=0.10$; the diluted pool had a volume of 113 ml and 6,561 $C'IA_{50}$/ml. It was then applied on a second column of CM cellulose (30×250 mm) equilibrated with 0.09 M NaCl in 0.02 M acetate buffer, pH 5.0, $\mu=0.10$. The column was washed with 0.14 M NaCl in 0.02 M acetate buffer, pH 5.0, $\mu=0.15$, until the effluent became free of both C'2 and C'7 activities. The column was then eluted with 1,000 ml of a linear gradient from $\mu=0.15$ to 0.26 of NaCl in 0.02 M acetate buffer, pH 5.0, at a flow rate of about 50 ml/hr, followed by a drive with 500 ml of 0.25 M NaCl in 0.02 M acetate buffer, pH 5.0, $\mu=0.26$. Fractions of about 12 ml were collected. As shown in Fig. 2, the peak of C'3 activity was separated well from both C'8 and C'9 activities.

Fractions 58 to 85 were pooled and adjusted to pH 7.5 with 0.1 M Tris [tris (hydroxymethyl) amino methane]. The pool was adjusted to a

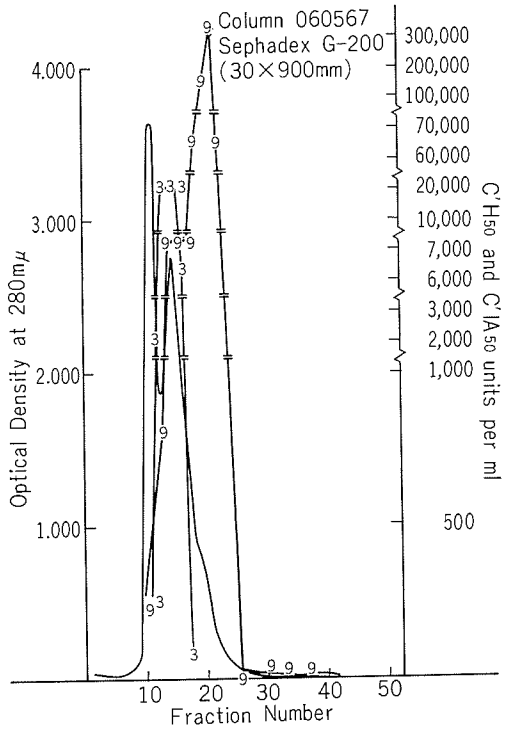

FIGURE 1 Separation of C'3 from 19S fraction and activity peak of C'9 on Sephadex G-200 column (First column of C'3 purification).

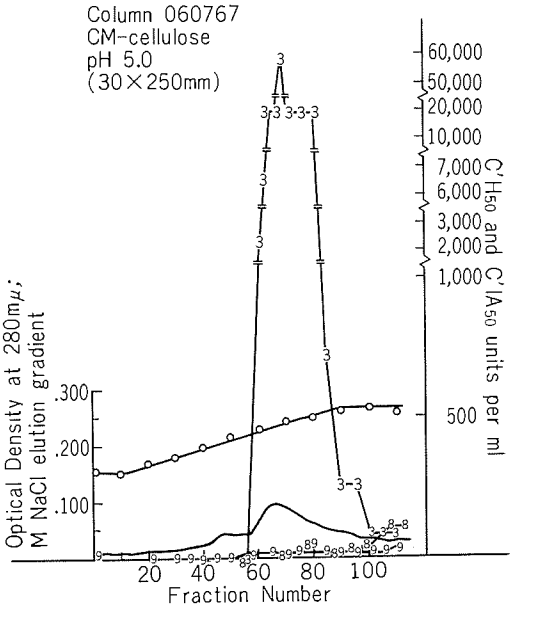

FIGURE 2 Elution chracteristics of guinea pig C'3 on CM-cellulose (Second column of C'3 purification).

conductance equivalent to $\mu=0.04$ by adding ice cold 0.005 M Tris HCl buffer, pH 7.5. It's volume was 2,574 ml and it showed 500 $C'IA_{50}$/ml. It was then applied to a third column of DEAE-cellulose (15 × 400 mm) equilibrated with 0.015 M NaCl in 0.005 M Tris HCl buffer, pH 7.5, at a flow rate of about 150 ml/hr. After its application, the column was washed with 0.04 M NaCl in 0.005 M Tris HCl buffer, pH 7.5, $\mu=0.045$, until the effluent became free from both C'6 and C'8 activities. It was then eluted with 1,000 ml of a linear gradient from 0.04 M to 0.2 M NaCl in 0.005 M Tris HCl buffer, pH 7.5, $\mu=0.045$ to 0.21, at a flow rate of about 30 ml/hr. Fractions of about 10 ml were collected. As shown in Fig. 3, only C'3 activity was detected and this coincided with the major protein peak. Fractions 37 to 48 were pooled and concentrated to about 9 ml by ultra-filtration in the cold. The C'3 preparations of this step were shown to contain no detectable activity of any complement component other than C'3, but they usually showed several protein zones by disc-electrophoresis, as shown in Fig. 5.

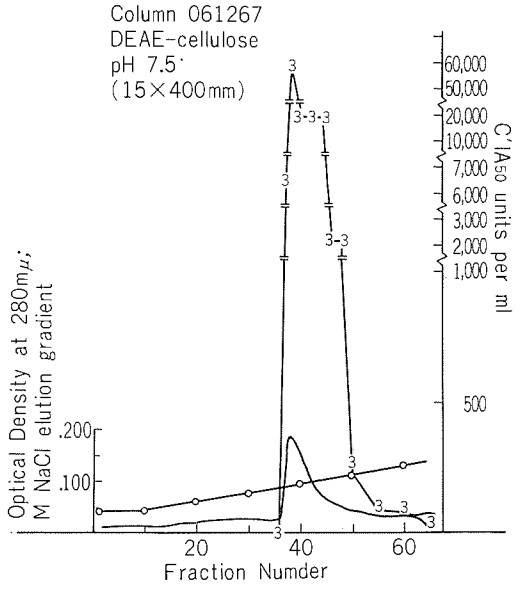

FIGURE 3 Elution characteristics of guinea pig C'3 on DEAE-celulose (Third column of C'3 purification).

The concentrated pool was applied to a fourth column of Sephadex G-200 (30 × 900 mm) equilibrated with isotonic veronal-buffered saline (pH 7.4, $\mu=0.147$), and eluted with the same buffer. Fractions of about 10 ml were collected. As shown in Fig. 4, one major protein peak was separated from a small peak which appeared later. C'3 activity coincided with the major peak. Fractions 17 to 21 were pooled. The volume of the pool (C'3 #11) was 50 ml and it showed $OD_{280}=0.133$ and 39,400 $C'IA_{50}$/ml in this experiment. The protein yield was 3.54% of the euglobulin fraction and the yield of $C'IA_{50}$ activity was 592%. The apparent increase in activity at the second (CM-cellulose) column step must be due to removal of an inhibitor(s), which acts on either the C'2 or C'3 step.

2. *Electrophoretic analysis of purified C'3*

The preparation of purified C'3 was analyzed by

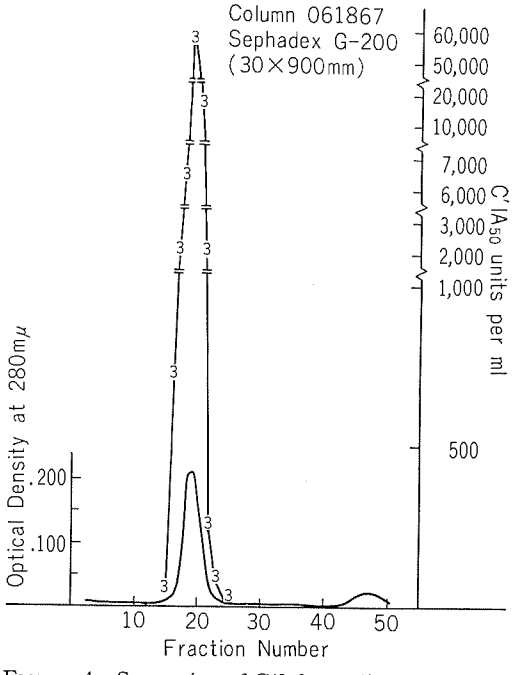

FIGURE 4 Separation of C'3 from albumin fraction (Fourth column of C'3 purification).

disc-electrophoresis. Samples of both the purified material (C'3 #11) and the pool of C'3 fractions from the third column were concentrated by ultra-filtration to give an optical density of about 1.0 at 280 mμ. As a control, one tenth dilution of whole guinea pig serum was run simultaneously.

As shown in Fig. 5, the purified C'3 preparation gave a single zone in the β_1 region and there was no detectable contaminating component, while the pool from the third column showed a diffuse zone in the albumin region as well as in the region of the β_1 component. Some of the latter fractions from other experiments also showed a few additional zones in the region from β to γ globulin.

FIGURE 5 Disc electrophoresis of guinea pig C'3. Left: 1/10 diluted fresh guinea pig whole serum; middle: purified C'3 preparation; right: pool of C'3 from the third column.

3. *Inhibition of hemolytic activity of C'3 by rabbit anti-C'3 antiserum*

As shown in Table 1, one drop of purified C'3 preparation #13 diluted in VBSS and one drop of anti-C'3 antiserum also diluted in the same buffer were delivered into each cup of a Microtiter plate. Control cups receiving a drop of VBSS instead of C'3 or antiserum were also included. The plate was incubated at 37°C for 20 minutes with continuous shaking. After incubation, one drop of partially purified C'2 diluted in VBSS, supplying about 200 effective molecules per cell, and one drop of EAC'1a,4 cells at a concentration of 1.0×10^8 cells/ml were delivered into every cup. After a further 25 min incubation at 37°C, one drop of a mixture of purified complement components containing sufficient C'5, C'6, C'7, C'8 and C'9 (C'5–9 reagent) was added. The plate was again incubated at 37°C for 90 min, and then centrifuged and read.

As shown in Table 1, rabbit anti-C'3 antiserum blocked the hemolytic activity of C'3 at higher concentrations.

4. *Inhibition of IA activity of C'3 by rabbit anti-C'3 antiserum*

Purified C'3 preparation #13 and anti-C'3 antiserum were diluted serially in VB-saline, and were delivered into cups of a Microtiter plate as in the previous experiment. After 20 min incubation at 37°C, one drop of partially purified C'2 diluted in VB-saline, supplying about 50 effective molecules of C'2 per cell, and one drop of EAC'1a, 4 at a concentration of 4.0×10^7 cells/ml were added. The plate was incubated at 37°C for 20 min with continuous shaking. One drop of human erytrocytes at a concentration of 1.0×10^8 cells/ml, was added to each cup. The plate was vibrated on a mechanical vibrator at 37°C for 15 min. It was then stood at 37°C for 60 min.

As shown in Table 2, the IA reactivity of C'3 was also inhibited by the anti-C'3 antiserum.

5. *Agglutination of EAC' 1a, 4, 2a, 3 cells by rabbit anti-C'3 antiserum*

Purified C'3 preparation #13 was diluted serially

TABLE 1 *Inhibition of hemolytic activity of C'3 by rabbit anti-C'3 antiserum*

Dilution of C'3 #13	×2	×4	×8	×16	×32	×64	×128	×256	×512	×1024	×2048	Ab(−)
C'3(−)	0	0	0	0	0	0	0	0	0	0	0	0
×128	0	0	0	0	0	0	tr	2	3	4	4	4
×64	0	0	0	0	0	tr	1	2	4	4	4	4
×32	0	0	0	0	0	1	2	2	4	4	4	4
×16	0	0	0	0	tr	2	2	4	4	4	4	4
×8	0	0	0	0	2	3	3	4	4	4	4	4
×4	0	0	0	2	2	3	4	4	4	4	4	4
×2	0	0	0	2	2	4	4	4	4	4	4	4

Dilution of anti-C'3 antiserum (#C-2)

Lysis was graded from 0 (no visible lysis) to 4 (complete lysis).
tr shows trace of hemolysis detected.

TABLE 2 *Inhibition of IA activity[a] of C'3 by rabbit anti-C'3 antiserum*

Dilution of C'3 #13	×2	×4	×8	×16	×32	×64	×128	×256	×512	×1024	×2048	Ab(−)
C'3(−)	tr	0	0	0	0	0	0	0	0	0	0	0
×512	tr	0	0	0	0	0	1	3	4	4	4	4
×256	tr	0	0	0	0	1	2	3	4	4	4	4
×128	tr	0	0	0	1	2	3	4	4	4	4	4
×64	tr	0	0	1	2	3	4	4	4	4	4	4
×32	tr	0	0	2	2	4	4	4	4	4	4	4
×16	1	1	1	2	3	4	4	4	4	4	4	4
×8	1	1	2	4	4	4	4	4	4	4	4	4

Dilution of anti-C'3 antiserum (#C-2)

a IA was graded from 0 (no visible adherence) to 4.
tr shows trace of adherence detected.

in VB-saline. One drop of each dilution was delivered into each cup of a row of a Microtiter plate by a pipette dropper. As controls, VB-saline was added to two rows of cups instead of C'3. One drop of partially purified C'2, supplying excess C'2 (about 500 effective molecules per cell) and one drop of EAC'1a,4 at a concentration of 1.0×10^8 cells/ml were added to every cup. The plate was incubated at 37°C for 15 min with continuous shaking. The cups then received one drop each of anti-C'3 antiserum diluted in VB-saline, as shown in Table 3, and the plate was vibrated at 37°C for 10 min, and then stood at 37°C for 60 min.

As shown in Table 3, EAC' 1a, 4, 2a, 3 cells were agglutinated by anti-C'3 antiserum, while EAC'1a, 4, 2a cells showed trace agglutination, probably due to a few sites containing C'3 on the cells used. The preparations of partially purified C'2 and EAC' 1a, 4 cells used had no detectable C'3 reactivity by routine IA titration. By the " cold method " for preparing EAC' 1a, 4 cells, however, it is possible to make a few SAC' 1a, 4, 2a, 3 sites on the cells, which can be detected by a high concentration of anti-C'3 antiserum but not by the routine IA method.

6. *Immunodiffusion analysis of the purified C'3 preparation*

On an Ouchterlony plate, fresh guinea pig

TABLE 3 *Agglutination of EAC′1a, 4, 2a, 3 by rabbit anti-C′3 antiserum*

Dilution of C′3 #13	×2	×4	×8	×16	×32	×64	×128	×256	×512	×1024	Ab(−)	Ab(−)
C′3(−)	1	tr	0	0	0	0	0	0	0	0	0	0
	1	tr	0	0	0	0	0	0	0	0	0	0
×512	2	1	tr	0	0	0	0	0	0	0	0	0
×256	2	1	tr	0	0	0	0	0	0	0	0	0
×128	2	2	tr	0	0	0	0	0	0	0	0	0
×64	4	3	1	tr	0	0	0	0	0	0	0	0
×32	4	4	3	1	0	0	0	0	0	0	0	0
×16	4	4	3	1	0	0	0	0	0	0	0	0

Dilution of anti-C′3 antiserum (#C-2)

Agglutination was graded from 0 (no visible agglutination) to 4.
tr shows trace of agglutination detected.

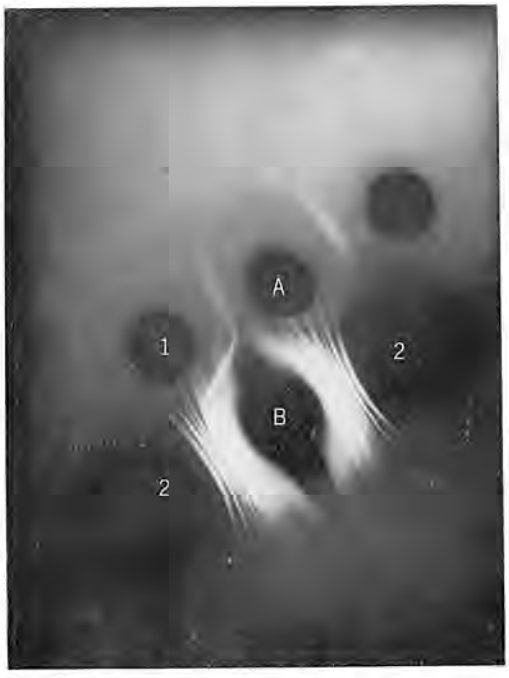

FIGURE 6 Ouchterlony's double diffusion analysis of guinea pig C′3. A: anti-purified C′3 antiserum; B: anti-guinea pig whole serum antiserum; 1: purified guinea pig C′3 preparation #13 (concentrated to $OD_{280}=0.50$ by ultrafiltration; 2: fresh guinea pig whole serum (1 : 1).

whole serum showed many lines against rabbit anti-guinea pig whole serum antiserum, as shown in Fig. 6, but only a single line against rabbit anti-C′3 antiserum. The purified preparation of C′3 #13 showed a single line against either antiserum.

The purified C′3 preparation #13 was also analyzed by immunoelectrophoresis. As shown in Fig. 7, the purified C′3 preparation gave only a single precipitation arc in the β_1 region against either anti-guinea pig whole serum antiserum or anti-C′3 antiserum. Treatment of the C′3 preparation at 56°C for 30 min did not cause any marked change in mobility of the component. On the other hand, guinea pig whole serum showed a faint but clear single arc against anti-C′3 antiserum in the β_1 region.

When the immunoelectrophoretic plate was covered with an agar layer containing EAC′ 1a,4 cells and excess amounts of C′2, C′5, C′6, C′7, C′8 and C′9, a clear hemolytic spot appeared with a sharp border superimposed on the precipitation arc, as shown in Fig. 8, while the heated C′3 preparation showed no hemolytic spot, in spite of the appearance of a distinct precipitation arc.

7. *Titration of C′3 by hemolysis*

C′3 can be titrated by immune adherence (NISHIOKA, 1963) or hemolysis. In the he-

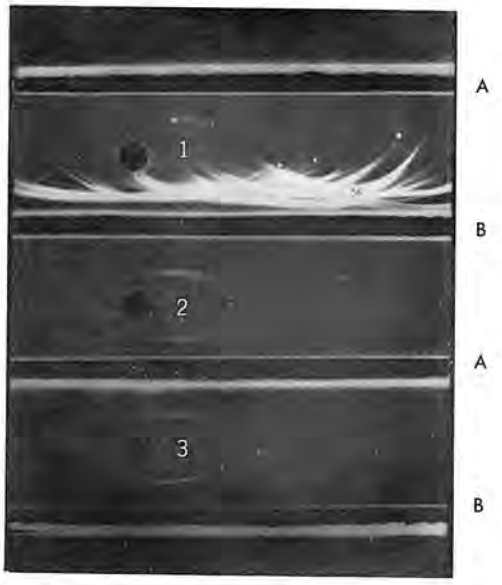

FIGURE 7 Immunoelectrophoretic analysis of guinea pig C'3. 1: fresh guinea pig whole serum; 2: purified guinea pig C'3 preparation #13; 3: purified guinea pig C'3 preparation #13 inactivated by heating at 56°C for 30 min. A: anti-purified C'3 antiserum; B: anti-guinea pig whole serum antiserum. The anode is to the right.

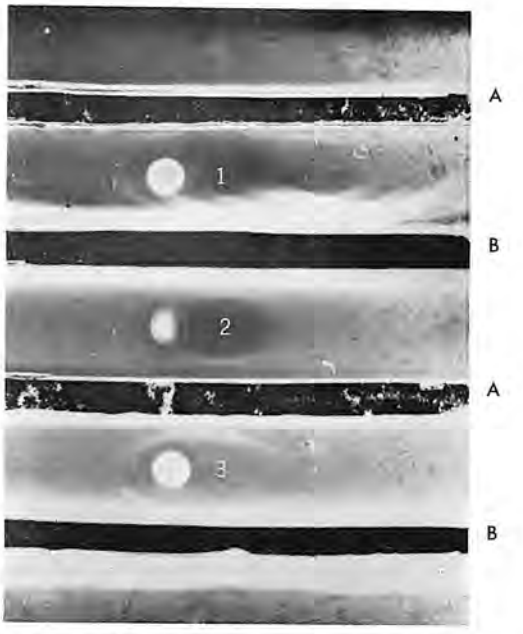

FIGURE 8 Combination of immunoelectrophoresis and hemolysis in agar. The antigens and antisera and their positions are the same as in Fig. 7. After development of precipitation arcs, the plate was covered with an agar layer containing EAC'1a,4 and moderate excesses of C'2, C'5, C'6, C'7, C'8 and C'9.

molytic method, several modifications are applicable using various intermediate complexes and various combinations of reagents, incubation temperatures and time schedules. To avoid complication from decays of various intermediates and to obtain relatively rapid completion of hemolysis, a two-step procedure was worked out for the titration of C'6 and C'7 by INOUE and NELSON (1965, 1966). In this method, the component to be titrated is incubated with EAC' 1a, 4 and an appropriate reagent to push the intermediate sites generated to the stable intermediate state of SAC' 1a, 4, 2a, 3, 5, 6, 7 at 30°C. A low incubation temperature is chosen to avoid rapid decay of labile intermediates. After the incubation, the reaction mixtures are mixed with reagent supplying moderate excess of C'8 and C'9 and incubated further at 37°C to complete lysis. With C'3, the same principle can be used routinely.

SAC' 1a, 4, 2a, 3 decays by two routes, one of which can be reversed by the addition of C'2 (INOUE and YONEMASU, 1968). Therefore, the effect of addition of C'2 and of the ionic strength of the medium were compared.

EAC'1a,4,2a cells were made by incubating EAC' 1a,4 cells with excess partially purified C'2 (about 300 effective molecules per cell) in VB-saline at 30°C for 7 min at a cell concentration of 5×10^7/ml. After centrifugation and washing the cells once with cold VB-saline, the cell suspension was divided into two portions, which were further washed twice in the cold with either VB-saline or VBSS, and then resuspended in the same buffer.

Various amounts of purified C'3 #18 were in-

cubated with 5.0×10^7 of EAC'1a,4,2a cells and a reagent (C'5–7 reagent), supplying moderate excesses of C'5, C'6 and C'7, in the absence or presence of partially purified C'2 (about 400 effective molecules per cell) either in VB-saline or VBSS at 30°C for 45 min in a volume of 2.0 ml. After incubation, 0.5 ml of a reagent (C'8–9 regent), containing excess C'8 and C'9, was added to each reaction mixture, and incubation was continued at 37°C for 90 min. After adding 5.0 ml of isotonic saline, the reaction mixtures were centrifuged and the optical density of the supernatant was read at 413 mμ. As controls, EAC'1a,4, 2a cells alone, cells with reagents C'5–7 and C'8–9 cells with C'3 (at the highest concentration) for cell blanks, and cells with distilled water for complete lysis were included.

As shown in Fig. 9, all the curves gave fairly straight line relationships between $-\ln(1-y)$ and the dose of C'3. The values of $-\ln(1-y)$ obtained in medium of low ionic strength, however, are far higher than those obtained at high ionic strength. Addition of C'2 enhanced the hemolytic response, because of the conversion of decayed sites SAC' 1a, 4 and SAC' 1a, 4, 3 to SAC' 1a, 4, 2a and SAC' 1a, 4, 2a, 3, respectively (INOUE and YONEMASU, 1968). For routine titration, therefore, a two-step procedure using EAC' 1a, 4 cells and excess C'2 in VBSS in the first step is recommended, and this method gave reliable and reproducible results.

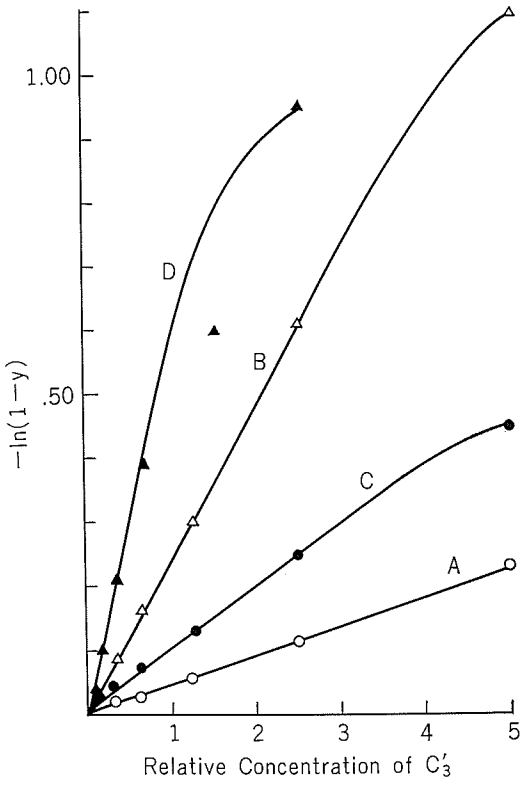

FIGURE 9 Titration of C'3 by two-step incubation method. A and C in VB-saline; B and D in VBSS; A and B without addition of free C'2; C and D with excess free C'2.

8. *Ionic strength and C'3*

It was clearly established that the hemolytic activities of whole complement as well as of some individual components were higher at low than at high ionic strength (RAPP and BORSOS, 1963; INOUE and NELSON, 1965, 1966). Therefore, the effect of the ionic strength of the reaction medium of the C'3 step was examined.

Media of different ionic strengths were prepared by mixing VB-saline and VB-sucrose, and the ionic strength was checked by measuring the conductivity of the medium. EAC'1a,4,2a cells were prepared as in the previous experiment in VB-saline, and centrifuged and washed once and resuspended in the same buffer. Portions of the cell suspension were then washed with 10 volumes of the buffers of different ionic strength and resuspended in the same buffers at a concentration of 1×10^8 cells/ml. Each suspension was mixed with an equal volume of purified C'3 diluted (1/150 of C'3 preparation #19) in the same buffer, and was incubated at 30°C for 15 min. After incubation, 1.0 ml of reaction mixture was poured into 10 volumes of cold buffer of the same ionic strength and centrifuged. The supernatant was discarded, and the tube was drained and wiped, and the cells were resuspended in 1.0 ml of VBSS. Then 1.0 ml of a C'5–7 reagent was added. After 45 min incubation at 30°C, 0.5 ml of a C'8–9 reagent was added to the reaction mixture and the mixture was incubated at 37°C for 90 min, as in the previous experiment.

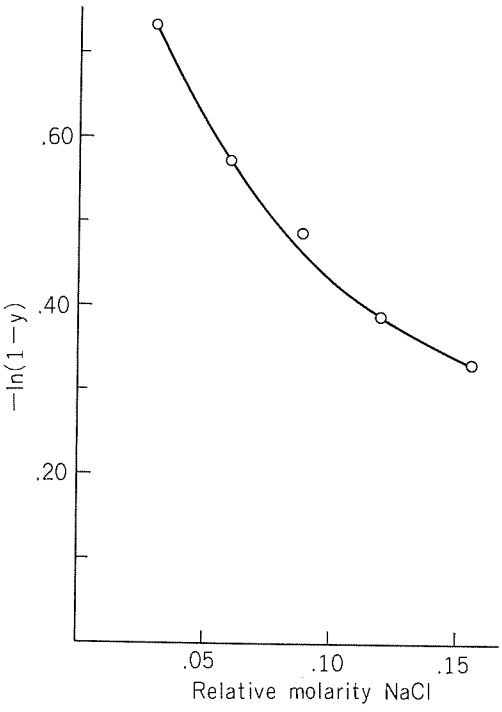

FIGURE 10 Relative reactivities of C'3 in media of various ionic strengths.

As shown in Fig. 10, the reactivity of C'3 was higher at low than at high ionic strength.

DISCUSSION

The method for isolating and purifying guinea pig C'3 described in this paper is very reproducible and gives a highly purified preparation with a high titer. It can also be easily modified for use with a larger volume of starting serum, because it uses only four serial chromtographies and one ultrafiltration step but does not include any technique to limit the volume of the preparation, such as block electrophoresis. The resulting preparation contains no detectable complement component other than C'3, and is highly pure judged from disc electrophoresis and immunodiffusion techniques. The combination of immunoelectrophoresis and hemolysis in agar has clearly demonstrated that the β_1-globulin present is actually C'3.

The C'3 activity of this purified preparation, however, is labile and is lost relatively rapidly, although it is relatively stable until the albumin fraction is removed by chromatography on the last column. Addition of gelatin at a concentration of 1% stabilizes the activity to some extent when the preparation is stored in a deep freezer. Various compounds alone and in combination are now being tested for their stabilizing activities.

Rabbit antiserum against purified C'3 gives only a single precipitation line against fresh guinea pig whole serum, and can inhibit both the hemolytic and the immune adherence reactivities of C'3. It also agglutinates the cells in the state of EAC' 1a, 4, 2a, 3 and EAC' 1a, 4, 3.

The dose-response curves obtained for C'3 using several reaction systems show that the one-hit theory (MAYER, 1961b) is also applicable to the C'3 step, in the guinea pig complement system at least. MÜLLER-EBERHARD *et al.* (1966), however, have shown that many molecules of human C'3 seems to be fixed around a single SAC' 1a, 4, 2a site. The discrepancy between these findings should be investigated further in relation to the observation by Rosse *et al.* that many holes are formed by human complement on the human erythrocyte membrane even when a limited proportion of the cells have been lyzed (ROSSE *et al.*, 1966), while the number of holes formed by guinea pig complement on sheep erythrocytes agrees with the value expected from $x=-\ln(1-y)$ (BORSOS *et al.*, 1964).

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