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SEPARATION OF C'4 FROM C'1 INACTIVATOR AND PURIFICATION OF BOTH SUBSTANCES[1]

SHINYA INAI, SEIICHI HIRAMATSU and KAZUYOSHI NAGAKI
The Center for Adult Diseases, Osaka Higashinari-ku, Osaka
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SUMMARY The separation of guinea pig and human C'4 from C'1 inactivator was carried out by various procedures. Highly purified human C'4 with potent hemolytic activity and negligible of C'1 inactivator activity was obtained. This purified human C'4 gave a single precipitin line against horse antiserum to whole human serum in the β_1-globulin region on immunoelectrophoresis. Antiserum prepared in rabbit against this purified human C'4 gave a single precipitin line against whole human serum on immunoelectrophoresis.

Purification of C'1 inactivator from human serum resulted in the isolation of a new protein which exhibited a single precipitin line against horse anti-whole human serum in the α_2-globulin region by immunoelectrophoretic analysis. The precipitin line of this protein was close to that of α_2-haptoglobin but further from the antibody trough than that of α_2-haptoglobin. This new protein, which seemed closely related to C'1 inactivator, was designated as α_{2I}-globulin.

INTRODUCTION

Since 1965, the interaction between EAC'1 cells and partially purified guinea pig C'4 has been investigated in our laboratory to obtain a satisfactory preparation of EAC'1,4 cells by sequential addition of partially purified C'1 and C'4 to sensitized sheep erythrocytes. In the course of the study evidence was obtained suggesting the presence of a substance in partially purified C'4 which might inactivate or destroy C'1 on sensitized sheep erythrocytes.

The inhibition of C'1-estrase activity by sera from several mammalian species has been extensively investigated by LEVY and LEPOW (LEVY and LEPOW, 1959). The inhibitor of C'1 esterase has been purified from human serum by PENSKY *et al.* using column chromatography (PENSKY, LEVY and LEPOW, 1961, LEPOW *et al.*, 1964), and the reaction between C'1-esterase inhibitor and C'1 on sensitized sheep erythrocytes has also been described (LEPOW and LEON, 1962; LEON and LEPOW, 1962).

Although the subtsance capabel of inactivating C'1 in partially purified C'4 seemed to be

1 Parts of this work were presented at the First Symposium on Complement in August, 1964 at Hakone, at the Third Symposium on Complement in July, 1966 at Hakone and at the 15th Annual Meeting of the Japanese Society of Allergy in October, 1965 at Tokyo, Japan.

identical to the serum inhibitor of C'1 esterase, these two substances have not yet been identified, so the former substance was tentatively designated as C'1 destroyer (NAGAKI *et al.*, 1967).

In 1966, in a report on methods for separation of nine components of guinea pig complement, general information on a substance which inactivated C'1 was given by NELSON *et al.*, and it was designated as C'1 inactivator (NELSON *et al.*, 1966). The elution pattern of C'1 inactivator from a DEAE cellulose column, described by NELSON *et al.*, was similar to that of C'1 destroyer in our study. For this reason, the substance which inactivated C'1 is designated as C'1 inactivator in this paper.

The present work was undertaken to eliminate contaminating C'1 inactivator from the C'4 preparation, and this was achieved by a combination of procedures, though not by any one procedure alone. This paper reports methods for separation of C'4 and C'1 inactivator from guinea pig and human sera, and some properties of purified C'4 and C'1 inactivator.

MATERIALS AND METHODS

1. *Intermediates of immune hemolysis*

Sheep erythrocytes (E), amboceptor (A), sensitized sheep erythrocytes (EA), the first component of guinea pig complement (C'1 gp), the second component of guinea pig complement (C'2 gp), EDTA treated guinea pig complement (C'-EDTA), EA with C'1 gp activity (EAC'1 gp cell) and EA with C'4 gp activity (EAC'4 gp cell) were prepared as described in previous reports (NAGAKI *et al.*, 1965, 1967).

2. *Titration of the fourth component of human complement (C'4 hu)*

This was done as described in the previous report (INAI *et al.*, 1967). The same method was applied to the titration of the fourth component of guinea pig complement (C'4 gp). The reciprocal of the highest dilution of the sample which produced one SAC'1,4 per cell was arbitrarily defined as the C'4 units (C'4H63) per ml of sample.

3. *Titration of C'1 inactivator in the test tubes*

Titration of the C'1 inactivator was performed as follows. To 0.5 ml each of serially two fold diluted samples was added 0.5 ml of optimally diluted C'1 gp so as to supply 2 effective molecules (eff. mol.) per cell. Controls consisted of 0.5 ml of C'1 gp of the same dilution and 0.5 ml of buffer. The mixtures were incubated for 30 minutes at 30°C. After incubation 0.5 ml of EAC'4 gp cells (1.5×10^8 cells per ml), was added to each tube which was incubated for a further 20 minutes at 30°C with constant shaking. Then 1.0 ml of C'2 gp diluted so as to supply 300 eff. mol. per cell was introduced into each tube, which was incubated for 30 minutes more at 30°C. Then each tube received 0.5 ml of C'-EDTA (1 : 12.5) and was quickly transferred to a water bath at 37°C for a further 60 minutes' incubation. Then 4.5 ml of cold 0.15 M NaCl were added to each tube, and the contents were thoroughly mixed and centrifuged.

The oxyhemoglobin contents of the supernatant fluids were measured by the optical density at 414 mμ by a Hitachi spectrophotometer using a cuvette of 1 cm light path. From the degree of lysis, y, $Z = -\ln(1-y)$ was calculated. Twice of the reciprocal dilution of a sample which showed half the Z value of the control tube was arbitrarily defined as one unit of C'1 inactivator.

4. *Microtitration of C'4 and C'1 inactivator activities*

Microtitration of C'4: A droplet (0.025 ml) of buffer was placed in each well of U-plate of Microtiter (Cooke Engineering Company) and suitable serial two fold dilutions of samples were made from ×2 to ×256 or ×4096.

To each well containing 0.025 ml of serially diluted sample, a droplet of EAC'1 cells (1.5×10^8 cells per ml) was added, and the mixture was mechanically agitated and incubated for 10 minutes at 30°C. Then each well received a droplet of C'2 gp suitably diluted so as to supply 150 eff. mol. per cell, and the mixture was mechanically agitated and incubated for 15 minutes at 30°C. Then a droplet of C'-EDTA (1 : 12.5) was added and the mixture was mechanically agitated and incubated for a further 60 minutes at 37°C. Then plates were centrifuged and the hemolysis was estimated as 0 (no hemolysis), 1+, 2+, 3+ and 4+ (complete homolysis).

Microtitration of C'1 inactivator: Serial two-fold dilutions of samples were made by the same method as for microtitration of C'4. A droplet of C'1 gp

diluted so as to supply 10 eff. mol. per cell was introduced into each well, and the mixture was mechanically agitated and incubated for 30 minutes at 30°C.

Then a droplet of EAC'4 gp cells (1.5×10^8 cells per ml) was added to each well which was mechanically agitated and incubated for 10 minutes at 30°C. Procedures for adding C'2 gp and C'-EDTA and reading hemolytic patterns were the same as those for microtitration of C'4.

5. *Preparation of ion-exchange celluloses*

Two batches of DEAE cellulose (lot No. 1518 and lot No. 1803, Brown Co.) were used throughout these studies. Celluloses were suspended in 1 N NaOH (5 liter of 1 N NaOH per 100 gm of DEAE cellulose), stirred for one hour and drained on a funnel with suction. Then the cellulose was washed successively with de-ionized water, 1 N HCl, deionized water and 1 N NaOH, and finally with de-ionized water until the cellulose was free of NaOH. Washed cellulose was suspended in the starting buffer, and the pH was adjusted with 1 N HCl. And the mixture was stirred for an hour, rewashed once with the starting buffer of the individual experiment and suspended in the same buffer. No difference was noticed between the two lots of DEAE cellulose in their capacities to separate C'4 from C'1 inactivator.

CM-Sephadex C-50 (lot No. 6648 and lot No. 8666, Pharmacia) were allowed to swell in de-ionized water for 24 hours, drained on a funnel with suction, washed successively with 0.5 N HCl, de-ionized water, and 0.5 N NaOH, rinsed with de-ionized water until free of NaOH, suspended in the starting buffer and adjusted to the required pH with 1 N HCl. CM-Sephadex C-50 were then washed once with the starting buffer and suspended in the same buffer.

6. *Block-electrophoresis*

Pevikon C-870 (Stockholm Superfosfat Fabriks A.B.) was washed once with de-ionized water, once with 0.05 M acetic acid and four times with de-ionized water. Then it was suspended in veronal buffer (80 ml of veronal buffer for 120 gm of Pevikon C-870) of 0.055 ionic strength and pH 8.6. This mixture was poured into a 6×33 or 6×22 cm tray (120 gm of Pevikon to 6×33 cm tray and 80 gm of Pevikon to 6×22 cm tray), to the ends of which 15 cm long towels had been fitted.

Excess buffer was allowed to drain away through the towels. The final block was about 0.6 cm in thickness. A 1.0 mm wide slit was cut across the block and approximate 1.5 ml of a sample was delivered into the slit by a syringe fitted with a needle. Then, the block was placed between two chambers of veronal buffer of 0.055 ionic strength and pH 8.6 in a cold room (2°C) and the ends of towels were dipped into this buffer. After a period of about 15 minutes equilibration, the slit was closed with a spatula.

A constant voltage of 4.5 V per cm was supplied for 18 to 20 hours. Then, 1.0 cm wide cross sections of the block were cut and eluted with 6.0 ml of 0.15 M saline.

7. *Estimation of protein*

Protein concentrations were estimated from the optical density at 280 mμ in a Hitachi spectrophotometer with a cuvette of 1 cm light path. Protein concentrations in some samples, especially in fractions obtained from the Pevikon block electrophoresis, were estimated with Folin phenol reagent (LOWRY *et al.*, 1951), and optical densities were read in a Hitach spectrophotometer at 750 mμ.

8. *Crude preparation of the fourth component of guinea pig complement* (C'4 gp)

A 8×60 cm column was set up containing 40 gm of DEAE cellulose of pH 7.4 in 0.08 M NaCl. Forty ml of guinea pig sera were adjusted to pH 5.8 with 0.15 N HCl, dialyzed overnight against 2 liter of cold de-ionized water, centrifuged and the NaCl concentration of the supernatant was adjusted to 0.08 M with 3 M NaCl, and to pH 7.2 with 0.08 N NaOH. This supernatant was applied to the column, which was washed with 2 liter of 0.08 M NaCl and 1 liter of 0.10 M NaCl, and then followed by 0.40 M NaCl. Active fractions eluted with 0.40 M NaCl were collected, pooled and stored at −20°C in divided portions.

9. *Immunoelectrophoresis*

Ten ml of 1% Oxoid Ionagar No. 2 in veronal HCl buffer (μ=0.05, pH 8.6) was layered over photographic glass slide (7.5 cm × 12 cm) and then allowed to gelatinize. An antigen well (diameter 2 mm) was punched out 3 cm from the cathodal end. This well was filled with pooled human sera or C'4. Electrode reservoirs contained veronal HCl buffer (μ=0.05, pH 8.6). Electrophoresis

was carried out at 4°C, for 1.5 hours at 6 v/cm. Under these conditions, the spot indicating the albumin fraction moved to the anodal side about 2.5 cm from the antigen well while the β_1-globulin fraction did not move.

After electrophoresis, an antibody trough (2 mm width) was made with a razor blade. The distance between the edge of the trough and the well was usually 4 mm. Horse antiserum to human serum was put into the trough, and the slide was then usually kept overnight in a moist chamber at room temperature until precipitin lines could be seen. The glass slide was washed for 1 day with excess physiological saline solution, dried at 37°C and stained with 0.1% Amidoblack 10 B solution.

10. *Horse antiserum to human serum*

Horse antiserum to human serum produced by Osaka Microbial Diseases Research Foundation (Lot No. 2), was used for immunoelectrophoresis and Ouchterlony's double diffusion technique.

Absorbed horse antiserum to human serum was prepared as follows: Horse antiserum to human serum was absorbed with fractions of human serum devoid of C'4 hu, C'1 inactivator and β_{1C}-globulin.

When pseudoglobulin of human serum was applied to a DEAE cellulose column equilibrated with 0.09 M NaCl in 0.01 M Tris-HCl buffer pH 7.4, most of the serum protein except C'4 hu, C'1 inactivator and β_{1C}-globulin passed through the column. Appropriate volume of concentrates of this fraction was carefully added to antiserum to avoid the presence of excess antigens.

RESULTS

1. *Detection of C'1 inactivator in a crude preparation of C'4gp*

When 200 eff. mol. per cell of a crude preparation of C'4gp obtained as described in Materials and Methods were added to EAC'1gp cells, the Tmax (at which time SAC'1,4,2 reached its maximum) of resulting EAC'1gp 4gp cells was prolonged with increase in the reacting time between EAC'1gp cells and the crude preparation of C'4gp. Thus there seemed to be some substance in the crude preparation of C'4gp which was responsible for the prolongation of Tmax. To study this, tests were made to see whether this substance inactivated the hemolytic activity of C'1gp.

1) Interaction between C'1 on sensitized sheep cells and crude C'4gp

To investigate the effect of crude C'4gp on limited C'1gp on EA, EA with about 3 eff. mol. per cell of C'1gp was allowed to react with various amounts of crude C'4gp. The reaction time was varied and lysis of these cells was estimated with sufficient amounts of C'2 and C'-EDTA.

To four tubes, each containing 5.0 ml of EA (1.5×10^8 cells per ml), were added 5.0 ml of optimally diluted C'1gp ($\times$3,000) and tubes were incubated for 15 minutes at 30°C to allow sensitization. Then, 5.0 ml of a crude preparation of C'4 gp were added to each tube to give 50, 100, 400 and 1000 eff. mol. per cell and tubes were reincubated at 30°C. At suitable intervals, 1.5 ml samples were withdrawn from each tube, introduced into tubes each containing 1.0 ml of C'2 gp, diluted to supply 300 eff. mol. per cell and incubated for 30 minutes at 30°C. Then, 0.5 ml of C'-EDTA (1: 12.5) was added to each tube and tubes were transferred to a water bath at 37°C for a further 60 minutes. Then 4.5 ml of cold 0.15 M NaCl were added to each tube with thorough mixing and tubes were centrifuged. The oxyhemoglobin contents of the supernatants were measured at 414 mμ in a Hitachi spectrophotometer.

As seen in Fig. 1, increase in the amount of crude preparation of C'4 gp added and prolongation of the incubation time with C'4 gp caused a marked decrease in the hemolytic activity of C'1 gp on EA.

2) Decrease in the hemolytic activity of C'1 gp by a crude preparation of C'4 gp

The decrease in the hemolytic activity of C'1 gp on mixing it with a crude preparation of C'4 gp was measured with EAC'4 gp cells, and a sufficient amount of C'2 and C'-EDTA.

To 3 tubes, each containing 7.0 ml of optimally diluted C'1 gp, ($\times$8,000) were added 7.0 ml of an optimally diluted ($\times$2,000) crude preparation of C'4 gp. Three more tubes,

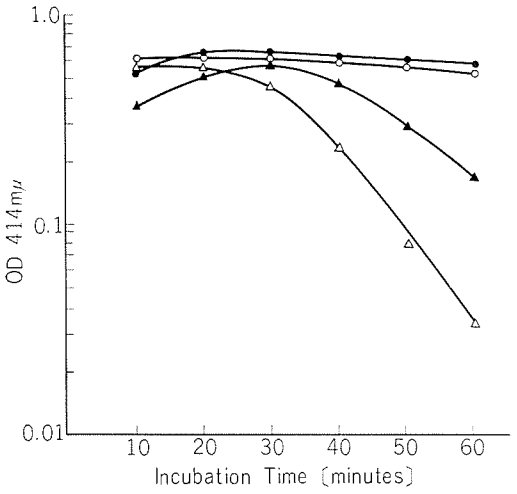

FIGURE 1 Interaction between C'1 gp on sensitized sheep erythrocytes and crude C'4 gp. EA with limited C'1 gp was allowed to react with 50 to 1,000 eff. mol. per cell of C'4 gp. Lysis of these cells was estimated after various incubation times.

●——● 50 eff. mol./cell of C'4
○——○ 100 eff. mol./cell of C'4
▲——▲ 400 eff. mol./cell of C'4
△——△ 1000 eff. mol./cell of C'4

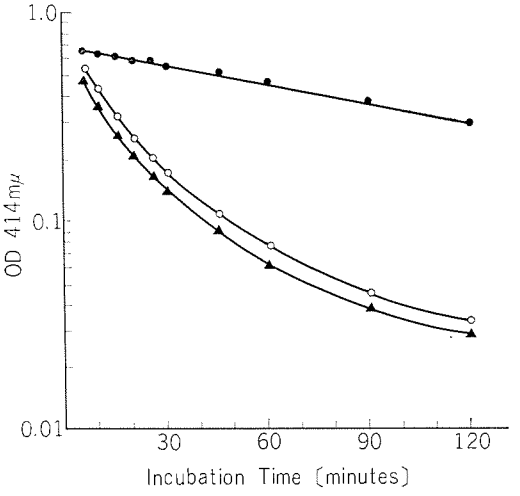

FIGURE 2 Decrease in the hemolytic activity of C'1 gp by mixing C'1 gp with crude C'4 gp. Reactions between C'1 gp and C'4 gp were carried out at 0°C, 30°C and 37°C. Decrease in the hemolytic activity of C'1 gp was estimated with EAC'4 gp cells.

●——● Reaction at 0°C
○——○ Reaction at 30°C
▲——▲ Reaction at 37°C

each containing 7.0 ml of C'1 gp of the same dilution and 7.0 ml of veronal buffer, were set up as controls. These two series of tubes were incubated at 0°C, 30°C and 37°C. At suitable intervals 1,0 ml of samples from each tube were introduced into tubes containing 0.5 ml of EAC'1, 4 gp cells (1.5×10^8 cells per ml). These tubes were incubated at 30°C for 15 minutes, and then received 1.0 ml of C'2 gp diluted to supply 300 eff. mol. per cell. After further incubation at 30°C for 30 minutes, 0.5 ml of C'-EDTA (1: 12.5) was added to each tube which was immediately transferred to a water bath at 37°C and incubated for 60 minutes more. Then, 4.5 ml of cold 0.15 M NaCl were added to each tube with thorough mixing and tubes were centrifuged.

The oxyhemoglobin contents of the supernatants were measured at 414 mμ. As seen in Fig. 2, the hemolytic activity of C'1 gp gradually decreased on incubation, even at 0°C.

These results, indicate that this crude preparation of C'4 gp contained a substance which inactivated or destroyed the hemolytic activity of C'1 gp.

For titration of C'1 with EA and for preparation of EAC'1, 4 cells with sufficient SAC'1, 4 from EA, C'1 and C'4, it is necessary to obtain C'4 which is not contaminated with C'1 inactivator. Accordingly this separation was studied.

2. *Separation of human C'4 from C'1 inactivator by DEAE cellulose column chromatography*

Preliminary tests on the separation of C'4 from C'1 inactivator were done with guinea pig C'4. Rechromatographic separation of the crude preparation of C'4 gp on a DEAE cellulose column and by gelfiltration on a Sephadex G-200 column were attempted, but no satisfactory results were obtained. Subsequently, preparative zone electrophoresis of

crude C'4 gp was done using a Pevikon C-870 block, and considerable separation was obtained, though a small amount of C'1 inactivator activity still remained in the pooled active fraction of C'4 gp. Further separation of C'4 gp from C'1 inactivator was achieved using a CM-Sephadex C-50 column, at pH 5.5. When the crude preparation of C'4 gp was applied to this column, C'1 inactivator started to be eluted with 0.051 M NaCl in 0.01 M acetate buffer and maximum activity was detected in the fractions eluted with 0.13 M NaCl in 0.01 M acetate buffer. C'4 gp eluted with 0.21 M NaCl in the same buffer.

It seemed possible to separate C'4 gp from C'1 inactivator completely by combining the above methods, but C'4 gp activity is very labile. This difficulty could be counteracted by using a large amount of guinea pig serum initially to obtain C'4 gp uncontaminated by C'1 inactivator. However, since it is difficult to get large quantities of guinea pig serum, studies were made on human serum. C'1 inactivator similar to that in guinea pig serum was found in human serum.

As a starting material for the following experiments the pseudoglobulin fraction of human serum was used. Human serum were chilled, adjusted to pH 5.8 with 0.15 M HCl and put into a visking tube. After dialysis against de-ionized water for 15 hours at 4°C, a heavy precipitate was removed by centrifugation at 8,000 rpm for 15 minutes at 0°C. The supernatant was adjusted to the desired NaCl concentration by addition of 3 M NaCl and the pH was brought to 7.4 with NaOH.

DEAE cellulose column chromatography was tested as the first step in the separation of C'4 hu from C'1 inactivator. As described above, separation of C'4 gp from C'1 inactivator by DEAE cellulose column chromatography was not successful.

However a little difference between the elution patterns of C'4 hu and C'1 inactivator was observed on DEAE cellulose column chromatography using a salt gradient.

1) Preliminary experiments

One ml of pseudoglobulin, prepared as described above, was applied to a 1.0×19 cm column of DEAE cellulose which had been equilibrated with 0.05 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4.

The salt gradient in the buffer was gradually increased from 0.05 M to 0.20 M and fractions of 5 ml each were collected. Elution of C'4 hu started at approximately 0.12 M NaCl and that of C'1 inactivator at approximately 0.14 M NaCl. The maximum activities of C'4 hu and C'1 inactivator were observed in effluent fractions No. 27 to No. 29 and No. 33 to No. 35, respectively (Fig. 3, left).

Based on the above results, stepwise elution from the DEAE cellulose column was tested. A 1.0×19 cm DEAE cellulose column was equilibrated with 0.09 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4. After application of the sample the column was washed with 0.09 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4. Then 100 ml of buffer containing 0.13 M NaCl, pH 7.4, was applied to the column and fractions of 5 ml each were collected in an automatic fraction collector. After fraction No. 56 had been collected the elution fluid was changed to buffer with 0.18 M NaCl and again fractions of 5 ml each were collected.

As shown in Fig. 3 (right), most of the C'4 hu was collected in the effluent obtained with 0.13 M NaCl but these fractions were slightly contaminated with C'1 inactivator. Fractions No. 58 to No. 64 eluted with 0.18 M NaCl, contained the residual C'4 hu and most of the C'1 inactivator.

2) Large scale experiments

To eliminate the contaminating C'1 inactivator from C'4 hu, DEAE cellulose column chromatography was employed as the first step in a large scale experiment. It was considered that a small loss of C'4 hu was unavoidable with any procedure.

Approximately 400 ml of pseudoglobulin were applied to a 6×20 cm column of DEAE cellulose equilibrated with 0.09 M NaCl in 0.01 M

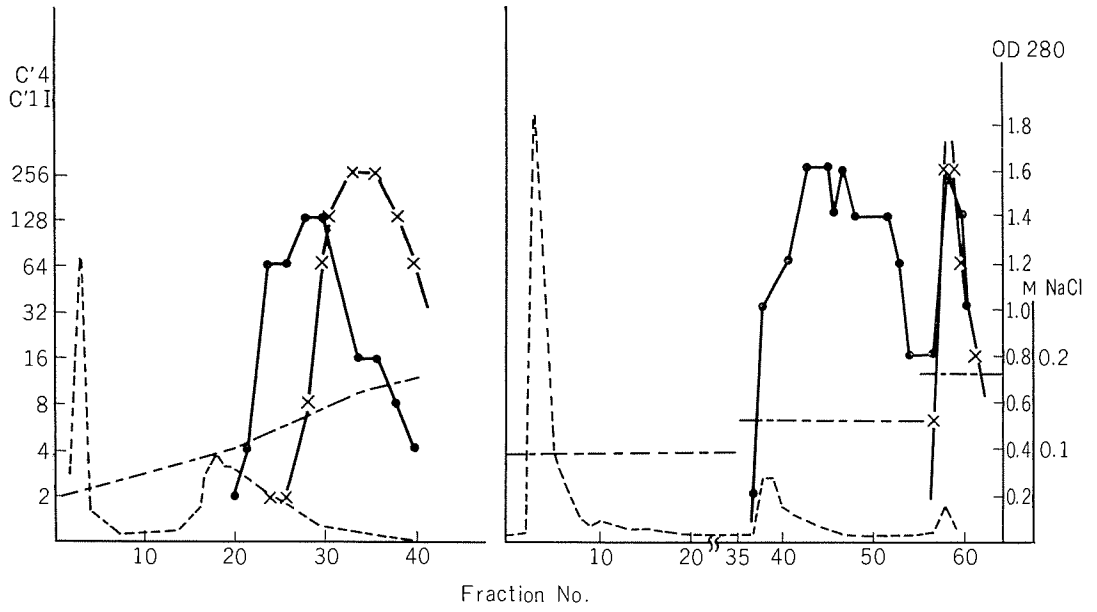

FIGURE 3 Elution characteristics of human C'4 and C'1 inactivator from DEAE cellulose column. The left figure shows the elution pattern of C'4 hu and C'1 inactivator with a salt gradient. The right figure shows the stepwise elution of C'4 and C'1 inactivator from a DEAE cellulose column.
●——● C'4 ×——× C'1 inactivator ●–·–·–● M NaCl ●- - - - -● OD 280

Tris-HCl buffer. The column was washed with 10 liter of chilled 0.09 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4. Then the column was eluted with buffer with 0.13 M NaCl and the first 300 ml of effluent were discarded. The next 2500 ml of effluent were collected. This fraction was concentrated to about 15 ml by ultrafiltration at 4°C. This fraction which was rich in C'4 hu was tentatively designated as DEAE-A and used for further purification of C'4 hu.

To purify C'1 inactivator, fractions containing C'1 inactivator were separated from pseudoglobulin as described below.

The procedures for preparation of pseudoglobulin, its application to the DEAE column and washing of the column with 0.09 M NaCl in Tris-HCl buffer were similar to those in the preparation of DEAE-A.

The fractions eluted with 0.13 M NaCl in Tris-HCl buffer were collected and elution was continued with 1,000 ml of 0.26 M NaCl in Tris-HCl buffer. The first 250 ml of effluent were discarded and the next 300 ml were collected. This fraction was dialyzed against de-ionized water for 2 hours at 0°C and concentrated to about 10 ml by ultrafiltration.

This fraction contained traces of C'4 and most of the C'1 inactivator of the starting material. This fraction was designated as DEAE-B and used for further purification of C'1 inactivator.

3. *Purification of C'4 hu*

1) CM-Sephadex Column chromatography

As with guinea pig C'4 and C'1 inactivator, CM-Sephadex C-50 column chromatography

* The activities of C'4 and C'1 inactivator in Figs. 3 to 10 show the highest dilution of the samples which gave 2+ hemolysis on titration of the two activities by the microtitration method. C'1 I in Figs. 3 to 10 indicates human C'1 incativator.

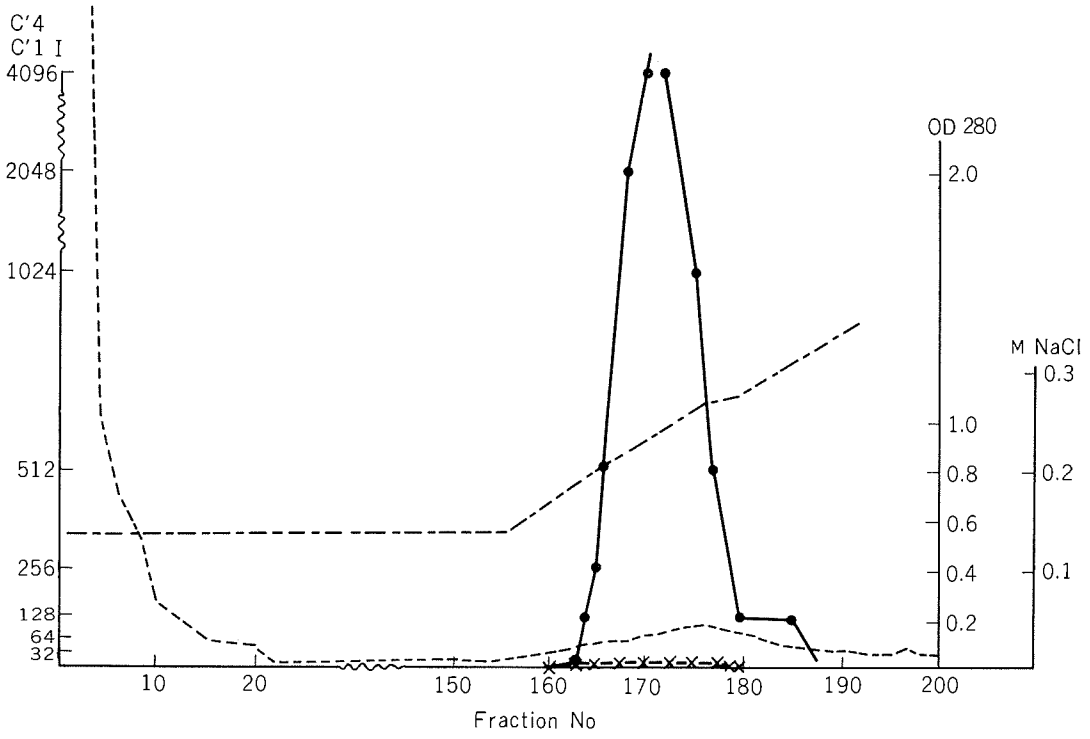

FIGURE 4 Purification of human C'4 from the DEAE cellulose column (DEAE-A) by CM-Sephadex C-50 column chromatography.

●——● C'4 ×——× C'1 inactivator ●-----● M NaCl ●-----● OD 280

was the most effective procedure for elimination of contaminating C'1 inactivator from C'4 hu.

As described in the next section, other contaminating serum proteins were eliminated by washing the CM-Sephadex column with 0.04 M NaCl in 0.01 M acetate buffer pH 5.5, and the elution peak of C'1 inactivator was at 0.12 M to 0.14 M NaCl in the same buffer.

Therefore, the next experiment was performed as follows: Approximately 15 ml of DEAE-A, adjusted to NaCl concentration of 0.12 M and pH 5.5, was applied to a 2×10 cm column of CM-Sephadex previously equilibrated with 0.13 M NaCl in 0.01 M acetate buffer pH 5.5. The column was washed with 1550 ml of the same buffer. Fractions of 10 ml were collected in an automatic fraction collector. After fraction No. 155 had been collected, the NaCl concentration was increased linearly from 0.13 M to 0.35 M. As shown in Fig. 4, the elution peak of C'4 hu was observed in fractions (No. 168 to 172) at 0.21 M NaCl. Fractions No. 166 to 176 were collected and concentrated to about 3 ml by ultrafiltration. This material was used for further purification of C'4.

2) Pevikon block electrophoresis

Pevikon block electrophoresis is also a useful method for separation of human C'4 and C'1 inactivator and for elimination of other contaminating serum proteins.

Partially purified C'4 hu, prepared by CM-Sephadex column chromatography as described above, was used for this procedure.

The Pevikon C-870 and the apparatus and buffer used in electrophoresis were described

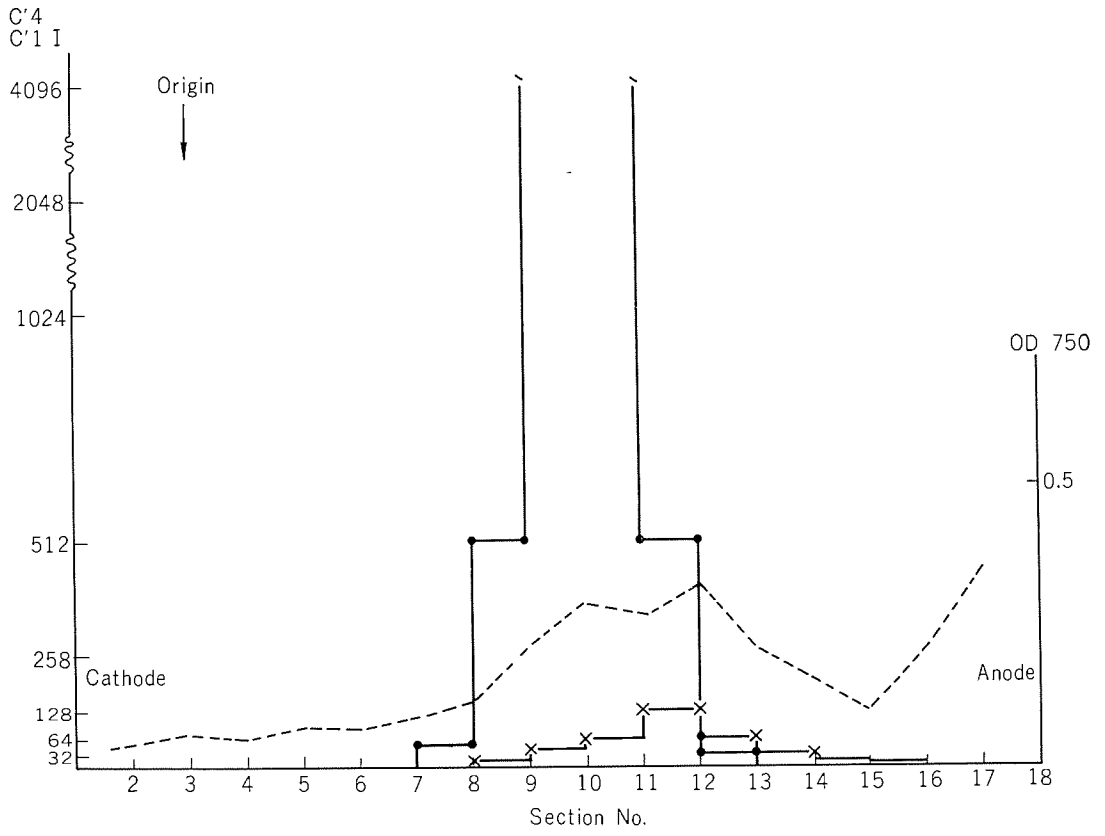

FIGURE 5 Purification of human C'4 from CM-Sephadex C-50 column by Pevikon C-870 block electrophoresis.
●——● C'4 ×——× C'1 inactivator ●-----● OD 750

in Materials and Methods. Approximately 3 ml of partially purified C'4 preparation was divided into two equal portions and each was applied to a $0.6 \times 6 \times 22$ cm block of Pevikon C-870 in veronal buffer pH 8.6, $\mu = 0.055$. Electrophoresis was carried out at 2°C for 20 hours, at 4.5 V/cm.

Then 1.0 cm wide cross sections of the block were cut and eluted with 6.0 ml of 0.15 M saline. C'4 hu and C'1 inactivator were detected by microtitration and the protein content of each section was estimated by Folin's method.

As seen in Fig. 5, the peak of C'4 activity was observed in the sections which were 3 to 4 cm further towards the cathode than the peak of C'1 inactivator. However separation of these two substances was not complete and there was a small overlap. Eluates from fractions containing C'4 and minute amounts of C'1 inactivator were pooled and concentrate to about 2 ml by ultrafiltration. This material was used in the final step for purification of C'4 hu.

3) Final step of purification of C'4 hu

For further purification of C'4 hu, DEAE cellulose column chromatography with a salt gradient was employed. Since, in the first step for purification, stepwise elution from a DEAE column with 0.13 M NaCl was used, other serum proteins eluted by a salt gradient

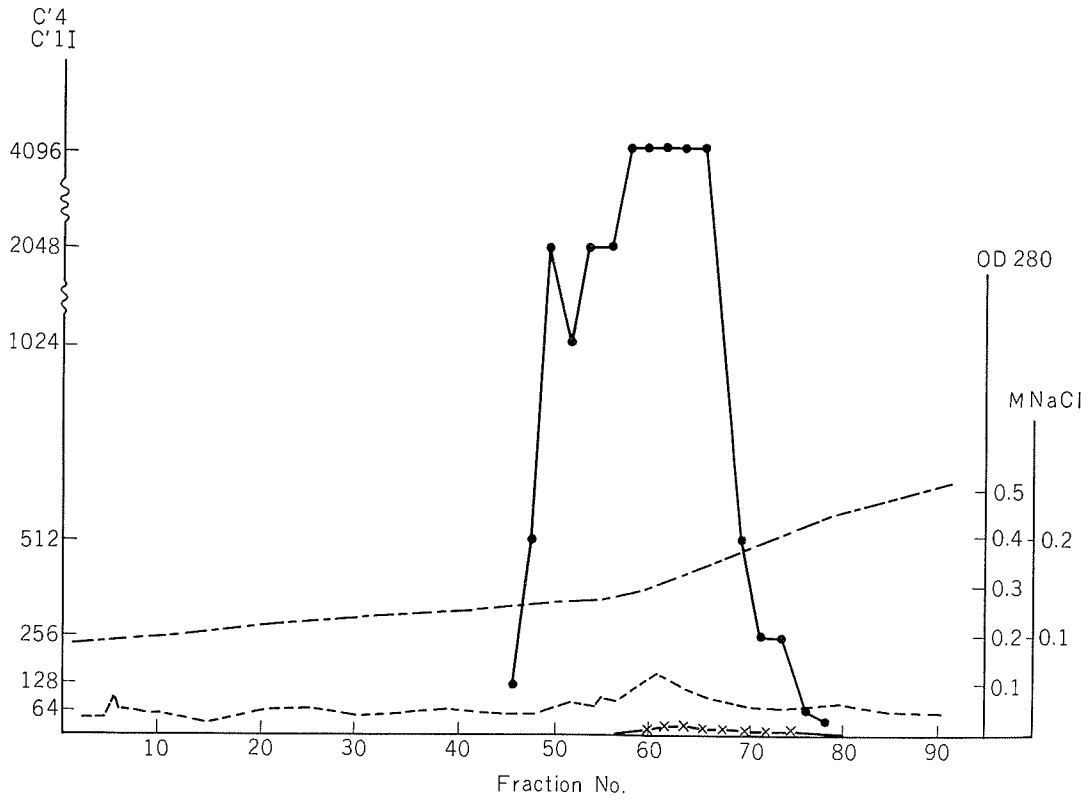

FIGURE 6 Final step for purification of human C'4 by DEAE cellulose column chromatography.
●——● C'4 ×——× C'1 inactivator ●—·—● M NaCl ●- - -● OD 280

from 0.09 M to 0.13 M were not eliminated.

Approximately 2 ml of concentrated material obtained by Pevikon C-870 block electrophoresis were applied to a 1×15 cm column of DEAE cellulose equilibrated with 0.09 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4. The NaCl concentration was gradually and linealy increased from 0.09 M to 0.25 M, and fractions of 5 ml were collected. As shown in Fig. 6, C'4 hu was eluted when the NaCl concentration reached 0.125 M and the elution peak was found in fractions (No. 58 to No. 66) at 0.150 M NaCl. Effluents containing most of the applied C'4 hu and minute amount of C'1 inactivator were collected and concentrated to about 1.5 to 2.5 ml by ultrafiltation. The properties of this highly purified C'4 hu are described in section 5.

4. *Purification of C'1 inactivator*

1) CM-Sephadex column chromatography

CM-Sephadex column chromatography was also employed as the second step for purification of C'1 inactivator

Approximately 10 ml of DEAE-B, adjusted to 0.04 M NaCl and to pH 5.5, was applied to a 2×10 cm column of CM-Sephadex C-50 equilibrated with 0.04 M NaCl in 0.01 M acetate buffer, pH 5.5. The column was washed with about 200 ml of 0.04 M NaCl in 0.01 M acetate buffer, and then the NaCl concentration was increased linealy from 0.04 M to 0.20 M.

Fractions of 10 ml were collected in an automatic fraction collector. As shown in

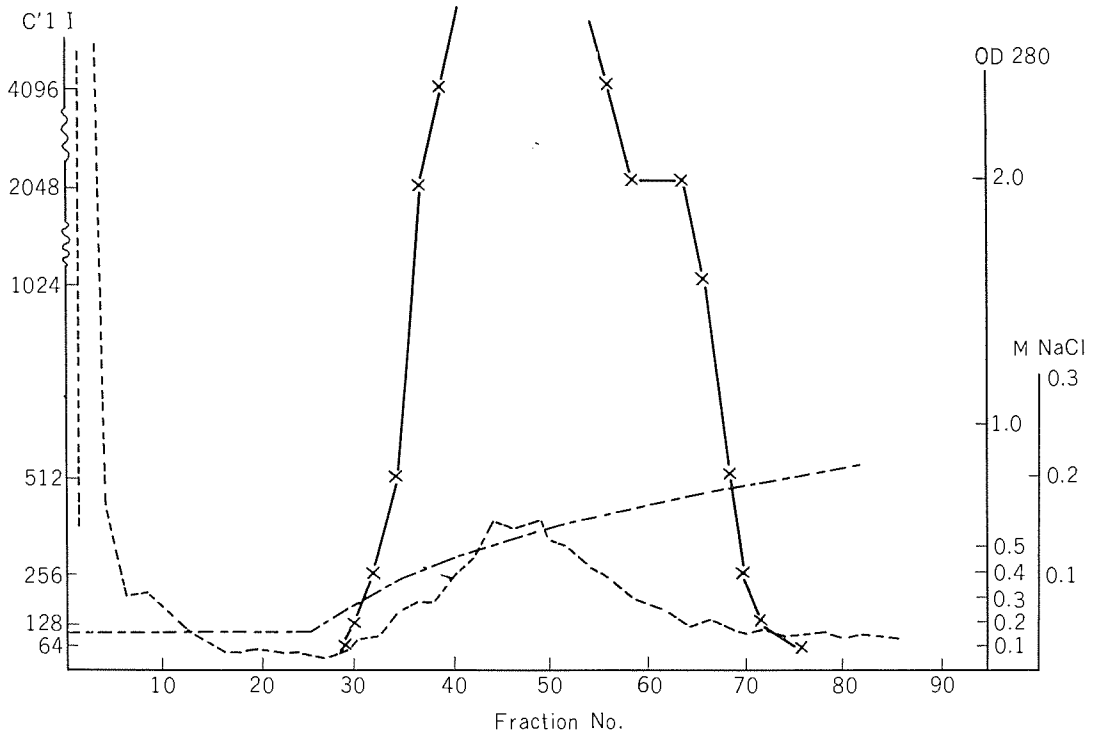

FIGURE 7 Purification of C'1 inactivator from DEAE cellulose column (DEAE-B) by CM-Sephadex C-50 column chromatography.

×———× C'1 inactivator ●—·—● M NaCl ●- - - -● OD 280

Fig. 7, the maximum activity of C'1 inactivator was found in fractions (No. 42 to No. 54) at 0.120 to 0.145 M NaCl. These fractions were collected and concentrated to about 2 ml by ultrafiltration. This preparation was used for further purification.

2) Pevikon block electrophoresis

Pevikon block electrophoresis was also used as the third step for purification of C'1 inactivator. Approximate 2 ml of C'1 inactivator, which had been partially purified by CM-Sephadex column chromatography, was applied to a $0.6 \times 6 \times 33$ cm block of Pevikon C-870 in veronal buffer, pH 8.6, $\mu = 0.055$.

The other conditions for electrophoresis and elution of C'1 inactivator from Pevikon were similar to those employed in purification of C'4 hu.

As shown in Fig. 8, the maximum activity of C'1 inactivator was found in the eluates from sections No. 19 to No. 22.

Active fractions (Section No. 17 to No. 22) with minute amounts of contaminating protein were pooled and concentrated to about 2 ml by ultrafiltration at 4°C. This material was used for further purification of C'1 inactivator.

3) Final step in purification of C'1 inactivator

As with C'4 hu, DEAE cellulose column chromatography with a salt gradient was used for further purification of C'1 inactivator.

Approximately 2 ml of concentrated C'1 inactivator after Pevikon block electrophoresis were applied to a 1×15 cm column of DEAE

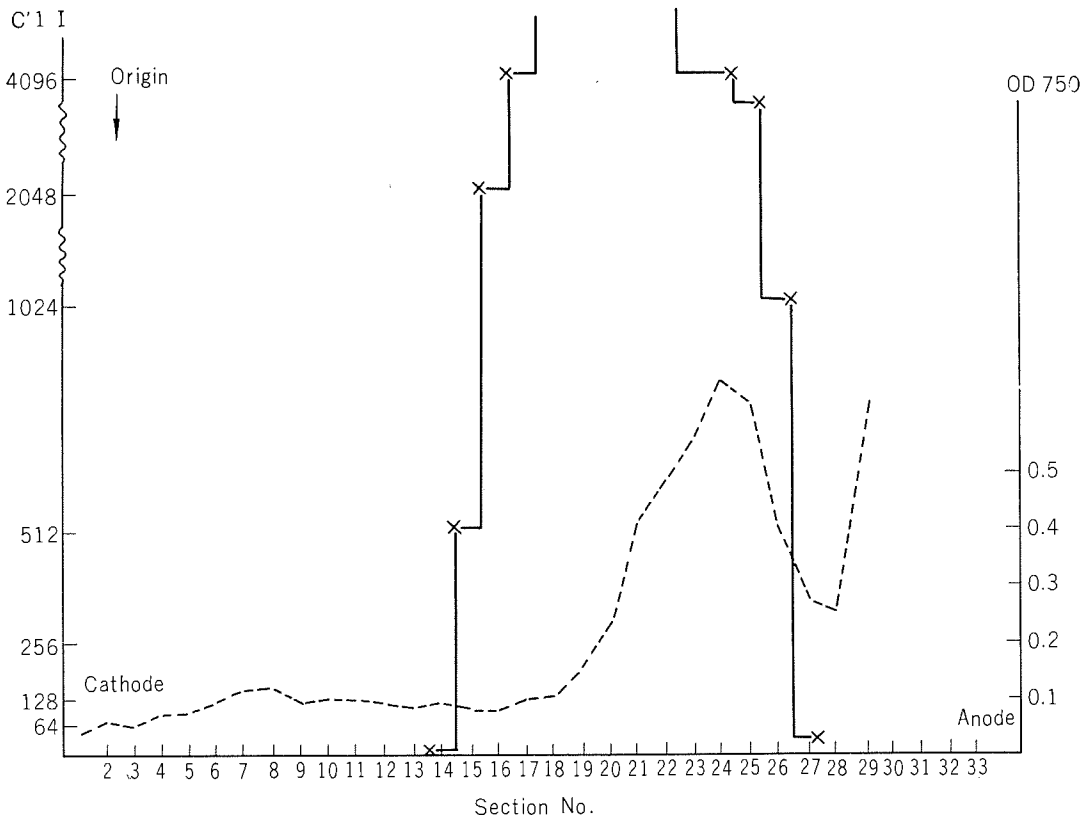

FIGURE 8 Purification of C'1 inactivator from CM-Sephadex C-50 column by Pevikon C-870 block electrophoresis. ×——× C'1 inactivator ●-----● OD 750

cellulose. The experimental conditions for equilibration of the cellulose and the salt gradient were similar to those used in purification of C'4 hu. As shown in Fig. 9, the elution peak of C'1 inactivator was in fractions (No. 66 to No. 68) with 0.175 M NaCl. Effluents in fractions No. 66 to No. 73 containing most of the C'1 inactivator and traces of other serum proteins were collected and concentrated to about 1.5 ml by ultrafiltration. This materials gave two or three precipitin lines against antiserum to whole human serum by immunoelectrophoresis.

Therefore, CM-Sephadex column chromatography with a salt gradient was repeated. Approximate 1.5 ml of concentrated purified C'1 inactivator were applied to a 1×20 cm column of CM-Sephadex. Except for the column size, the experimental conditions used in this experiment were the same as for the CM-Sephadex column chromatography of DEAE-B. Fractions of 5 ml were collected, and as seen in Fig. 10, maximum activity of C'1 inactivator was found in fractions No. 40 to No. 44. Effluents containing C'1 inactivator (Fractions No. 38 to No. 55) were collected and concentrated to about 0.5 ml by ultrafiltration. Some properties of the purified C'1 inactivator are described in section 5.

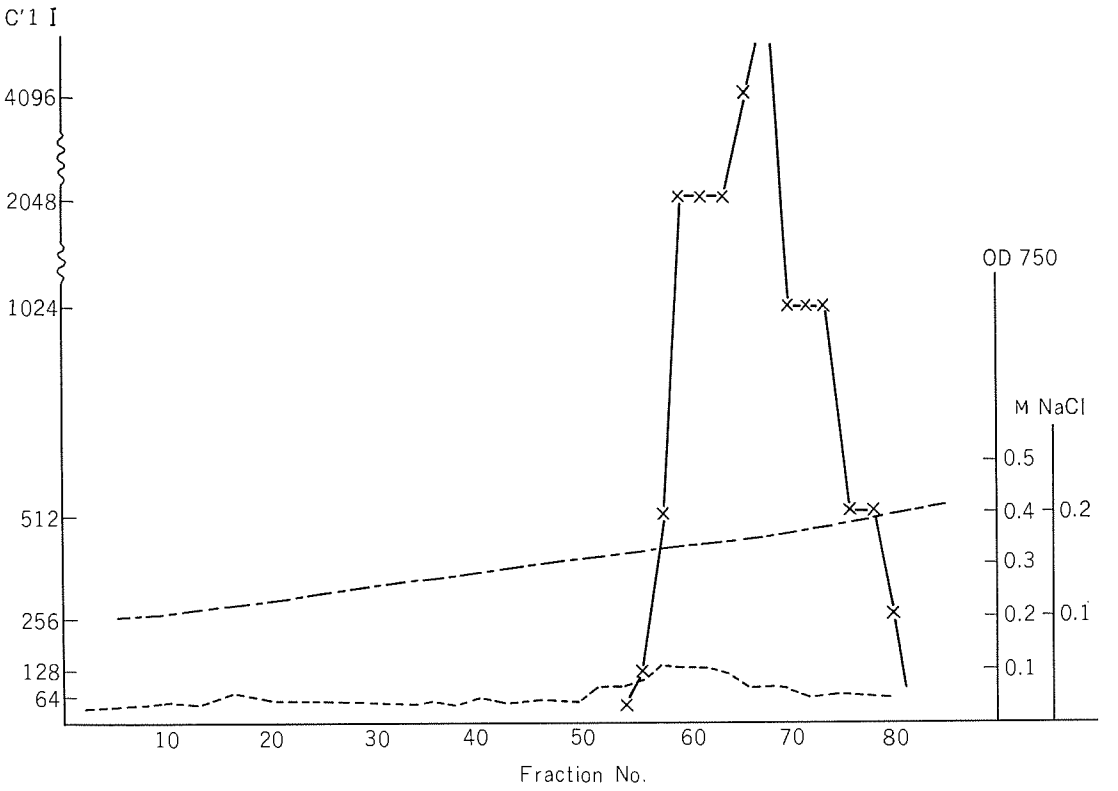

FIGURE 9 Final step for purification of C'1 inactivator by DEAE cellulose column chromatography.
×———× C'1 inactivator ●———● M NaCl ●- - - - - ● OD 750

5. *Characteristics of purified C'4 and C'1 inactivator*

1) C'4 hu

Highly purified human C'4 was obtained by the procedures for elimination of contaminating C'1 inactivator described above. The hemolytic activity, yield, protein content and activity of contaminating C'1 inactivator of three specimens of purified C'4 are sumarized in Table I. As shown in this table, the C'4 activity of one of these specimens (Preparation No. 2) was about 5 times higher than that of the original serum. For 63 per cent lysis of 7.5×10^7 EAC'1 cells approximately 0.003 μg protein of C'4 was needed. Contaminating C'1 inactivator was practically negligible in these specimens. The ratio of C'1 inactivator to C'4 H63 was 1 : 0.3 in the starting material, whereas, as shown in the table, this ratio was 1 : 1600 in the purest product. No other components of complement were detectable except traces of C'3 and C'7. As reported in the previous paper, satisfactory preparations of EAC'1 gp, 4 hu and EAC'1 hu, 4 hu cells with sufficient numbers of 1, 4 sites could easily be prepared with these purified C'4 and EAC'1 cells.

As shown in Fig. 11, on immunoelectrophoretic analysis of purified C'4 hu (Preparation No. 2 in Table I) a single precipitin line was obtained in the β_1-globulin region with horse antiserum to whole human serum. This precipitin line might be identical to the β_{1E}-

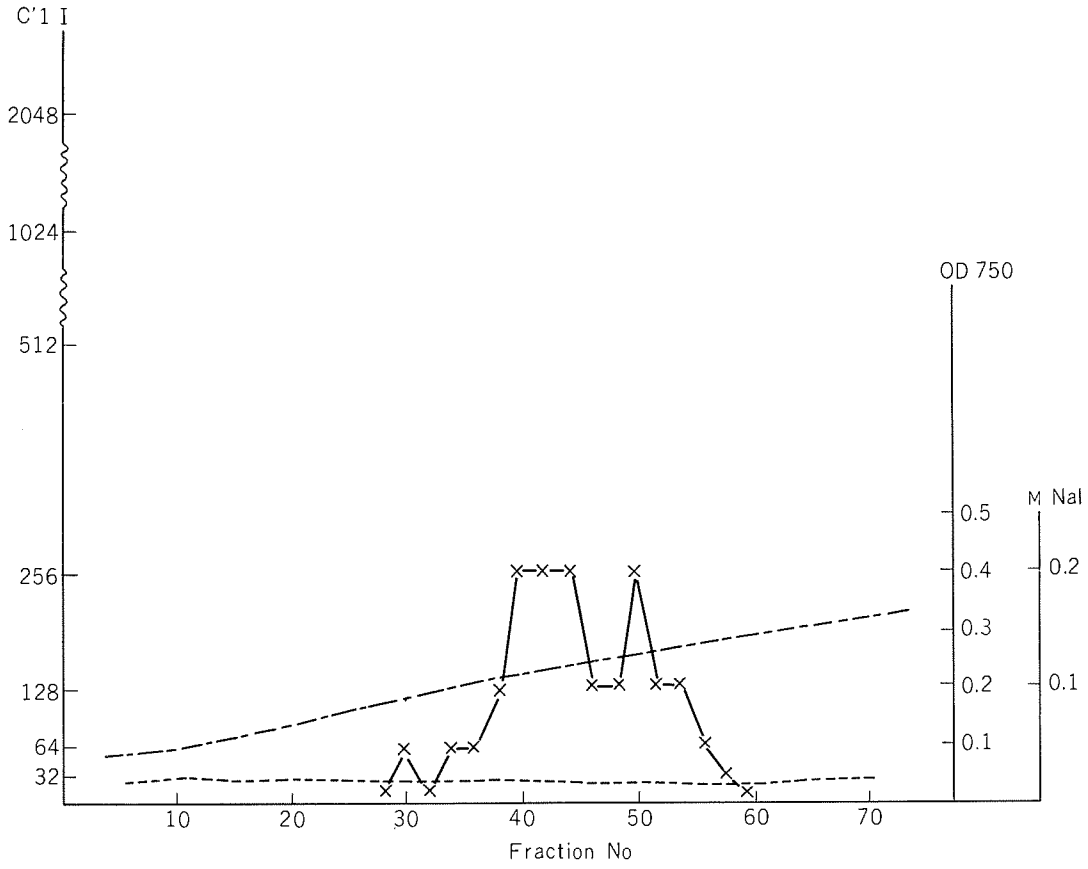

FIGURE 10 Rechromatography of purified C'1 inactivator on CM-Sephadex C-50 column.
×———× C'1 inactivator ●—·—● M NaCl ●- - - -● OD 750

TABLE 1 *Properties of purified C'4 hu*

Preparation	Volume ml	C'4H63 ($\times 10^{-4}$)	C'1 inactivator (u)	Protein mg/ml	Yield %	C'1 inactivator : C'4H63*
No. 1	1.9	50	905	1.5	5.0	1 : 520
No. 2**	2.25	62	388	2.0	3.0	1 : 1600
No. 3	1.2	32	250	1.6	2.0	1 : 1280

* This ratio was about 1 : 0.3 in the starting material.
** This was prepared from two 380 ml volumes of pseudoglobulin. Purification of each was started independently, and active fractions from the two parts were combined at the last step of purification.

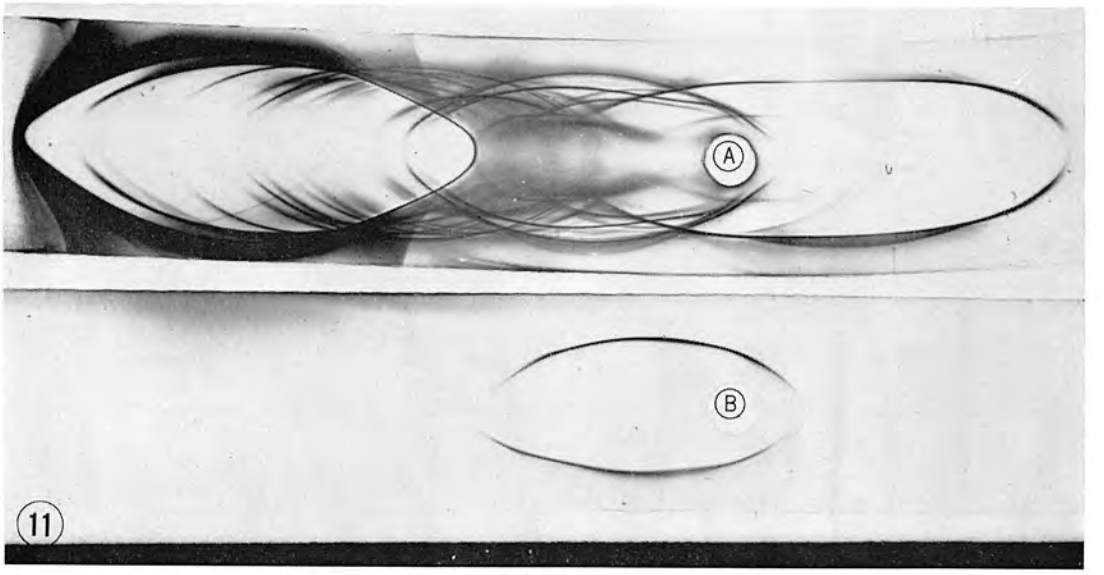

FIGURE 11 Immunoelectrophoretic pattern of purified C'4 hu. Purified C'4 hu, preparation No. 2 in Table 1, showed a single precipitin line against horse antiserum to human serum.
Well A: Whole human serum Well B: Purified human C'4
Troughs: Horse antiserum to human serum

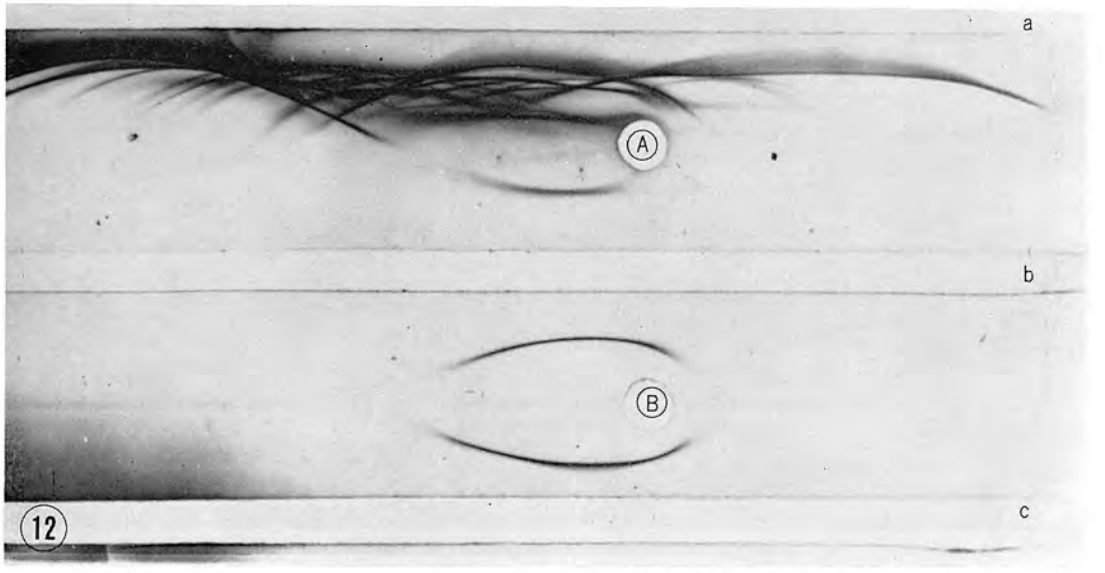

FIGURE 12 Immunoelectrophoretic pattern of whole human serum and purified C'4 hu against rabbit anti C'4 serum. Rabbit anti C'4 serum developed a single precipitin line not only to purified C'4 but also to whole human serum.
Well A: Whole human serum Well B: Purified human C'4
Troughs a and c: Horse antiserum to human serum
Trough b: Rabbit anti C'4 serum

globulin line reported by Müller-Eberhard and Biro (1963). Three rabbits were injected once with 1 mg of purified C'4 protein (Preparation No. 2) in complete Freund adjuvant. About a month later the antisera produced in these rabbits gave a sigle precipitin line of β_{1E}-globulin against whole human serum on immunoelectrophoresis (Fig. 12).

2) C'1 inactivator

The final material obtained on purification of C'1 inactivator was extremely potent in inactivating C'1. Thus, 1 ml of about 280,000 times dilution of this material could inactivate 1 eff. mol. of guinea pig C'1. The protein concentration in this material was 0.77 mg/ml. Therefore, approximately 0.0027 μg of protein could inactivate 1 eff. mol. of C'1 gp.

As shown in Fig. 13A, immunoelectrophoretic analysis of the purified C'1 inactivator showed a single precipitin line in the α_1-to α_2-globulin regin against antiserum to whole human serum. No reaction was observed with antiserum to α_2-macroglobulin or to α_2-haptoglobin. To identify the precipitin line of C'1 inactivator among the many precipitin lines of whole human serum developed against horse antiserum to whole human serum on immunoelectrophoresis, Ouchterloney's double diffusion technique was combined with immunoelectrophoresis. As shown in Fig. 13B, normal human serum and purified C'1 inactivator were subjected to electrophoresis by the method described in MATERIALS AND METHODS, and electrophoresed proteins reacted with horse antiserum to human serum and the absorbed horse antiserum to human serum. At the same time C'1 inactivator diffused in this plate. The precipitin line of C'1 inactivator in Ouchterloney's double diffusion technique fused with the line of C'1 inactivator which appeared on electrophoresis, and it also fused with one of the lines in the α_2-globulin region. Other lines in the α_1-globulin to α_2-globulin regions crossed the line of C'1 inactivator. The precipitin line, which fused with C'1 inactivator, was close to that of α_2-haptoglobin but further from the antibody trough than that of α_2-haptoglobin.

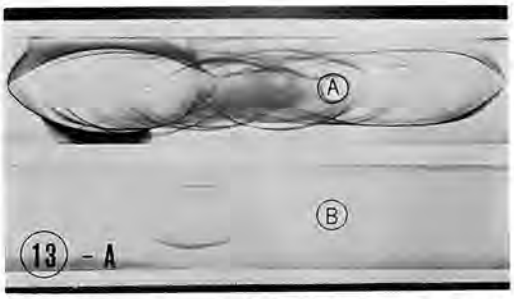

FIGURE 13 A Immunoelectrophoretic pattern of purified C'1 inactivator. Purified C'1 inactivator showed a single precipitin line against horse antiserum to human serum in the α_2-globulin region.
Well A: Whole human serum
Well B: Purified C'1 inactivator
Troughs: Horse antiserum to human serum

This new protein might be identical with C'1 inactivator itself, and it will be identified later.

DISCUSSION

For titration of the first component of complement, EA with C'4 activity (EAC'4 cell) has been used as a substrate, and EAC'4 cells were prepared from EAC'1, 4 cells by EDTA treatment. But in 1963 LEPOW *et al.* (1963) reported that the first component of human complement can be resolved into three activities by chromatography. It was found that one of these three substances, C'1q, was identical to the 11S component which was reported by MÜLLER-EBERHARD and KUNKEL (1961) and by TARANTA *et al.* (1961). According to MÜLLER-EBERHARD and KUNKEL (1961), the 11S component reacts with EA independently of Ca^{2+} and Mg^{2+}.

This led us to consider that C'1q was not dissociated from EAC'1, 4 cells by EDTA treatment. To examine this, R11S was prepared by the method of Müller-Eberhard and Kunkel and added to EAC'4 cells. Although lysis of EAC'4 cells was observed on addition of R11S, no hemolysis was found when R11S

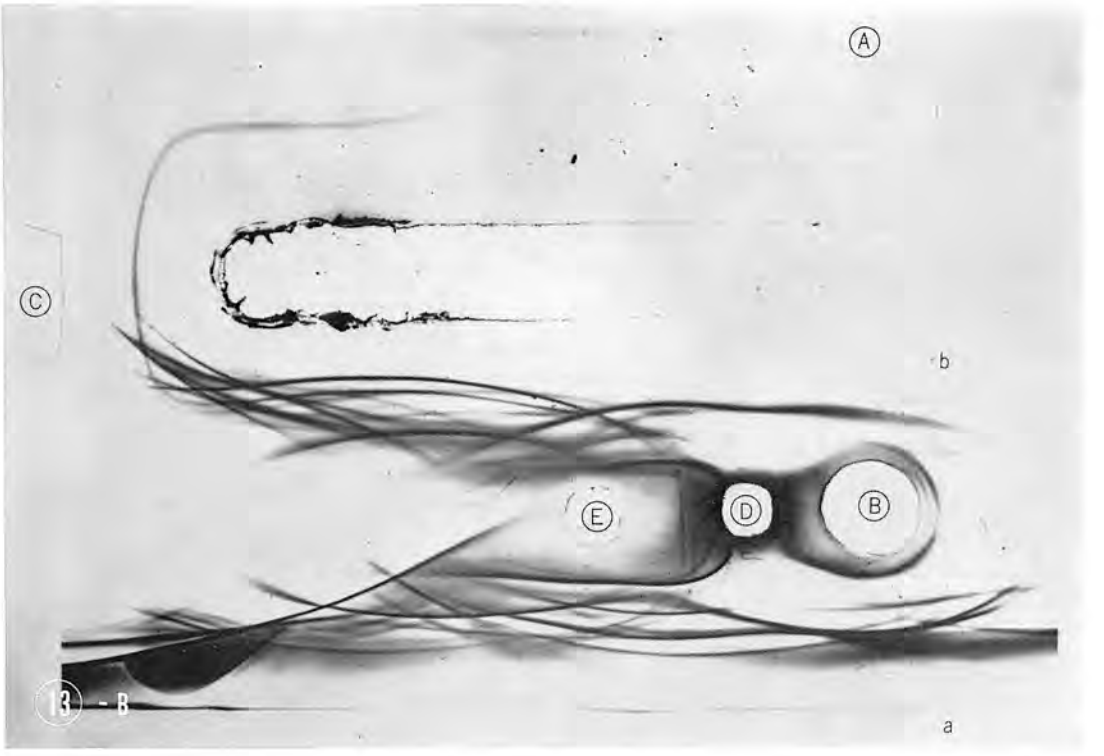

FIGURE 13 B Immunoelectrophoretic analysis of purified C'1 inactivator. C'1 inactivator and whole human serum were put in wells A and B, respectively. After electrophoresis, C'1 inactivator was put in well C, absorbed horse anti serum to human serum was poured into trough b and nonabsorbed horse antihuman serum was poured into trough a. The precipitin line formed between well C and trough b fused with one of the precipitin lines in the α_2-globulin region.

To detect the line of C'1 inactivator among the many lines formed between whole human serum and unabsorbed horse antiserum, absorbed antiserum and purified C'1 inactivator were poured into wells D and E, respectively. No precipitin lines formed between well D and E were extended enough to fuse that of C'1 inactivator formed between well B and trough b.

was added to EA or a mixture of EA and a crude preparation of C'4.

These results suggested that EAC'4 cells prepared from EAC'1, 4 cells by EDTA treatment had C'1q activity and were not theoretically true EAC'4.

Accordingly we planned to titrate C'1 using EA, sufficient C'4, C'2 and C'-EDTA. However, when C'1 was titrated with EA, crude C'4 gp, C'2 and C'-EDTA, the hemolytic activity of C'1 gp decreased with increasing amount of C'4 gp added. As was described in the results, the data of experiments on interaction between C'1 gp and crude C'4 gp suggest that some substance might be present in the crude preparation of C'4 gp which inactivated the hemolytic activity of C'1 gp.

In 1965, the presence of C'1 inactivator in a preparation of partially purified C'4 gp was reported by NELSON *et al.* (1965). It was thought to be identical to the C'1 esterase inhibitor of PENSKY *et al.* (1961).

Therefore, the term C'1 inactivator was also adopted for the substance which inactivated the hemolytic activity of C'1. However, in this study C'1 inactivator was not identified C'1 esterase inhibitor. First, efforts were made to exclude C'1 inactivator from the C'4 of guinea pig complement. However, it was difficult to obtain sufficient guinea pig serum for separation of C'4 from C'1 inactivator.

Accordingly, we had to use human serum in large scale experiments on the separation of C'4 from C'1 inactivator. MÜLLER-EBERHARD and BIRO (1964) succeeded in separating highly purified C'4 from human serum by TEAE cellulose column chromatography and Pevikon block electrophoresis, but they did not mention the possibility of contamination of their purified C'4 with C'1 inactivator. As described in the results, it was very difficult to separate C'4 from C'1 inactivator.

For example, guinea pig C'4 could not be separated from C'1 inactivator by DEAE cellulose column chromatography or Sephadex G-200 column chromatography. The elution peaks of human C'4 and C'1 inactivator on DEAE cellulose column chromatography were only slightly different. Pevikon block electrophoresis is a useful method for separation of guinea pig, or human C'4 from C'1 inactivator, but there was slight overlap of the two substances.

Another valuable method for sepration of C'4 from C'1 inactivator with both guinea pig and human material is CM-Sephadex column chromatography. In this case maximum activity of C'1 inactivator was found in fractions eluted at 0.125 M NaCl and that of C'4 was found at 0.210 M. But the fraction containing C'4 is slightly contaminated with C'1 inactivator.

Accordingly, to minimize the contamination from C'1 inactivator, these three methods were combined. The final product, obtained by four treatments to separate C'4 from C'1 inactivator, showed highly potent C'4 activity and contained negligible amounts of C'1 inactivator. As reported in the previous paper (NAGAKI *et al.*, 1967) satisfactory EAC'1, 4 cells could be prepared with this highly purified human C'4. This purified human C'4 gave a single precipitin line in the β_1-globulin region on immunoelectrophoretic analysis. Further immunoelectrophoretic analysis of human C'4 will be reported in the following paper (HIRAMATSU *et al.*, 1967).

C'1 inactivator could easily be purified using methods similar to those used for purification of human C'4.

Purification of C'1 inactivator from human serum gave a new protein which exhibited a precipitin line in the α_2-globulin region on immunoelectrophoretic analysis. This single precipitin line in the α_2-globulin region was not identical to either α_2-macroglobulin or α_2-haptoglobin, and was slightly further from the antiserum trough than that of α_2-haptoglobin.

The questions as to whether this new protein itself has activity to inactivate C'1 or whether it is a contaminant of highly purified C'1 inactivator must be studied. This new protein seems to be closely related to C'1 inactivator, and it was designated as α_{2I}-globulin.

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