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Studies on the Fourth Component of Complement
I. Titration of the Fourth Component of Complement in Human Serum

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SUMMARY

The method for titration of the fourth component of complement (C'4) developed by Hoffman was improved by changing the ionic strength of the medium and other conditions. The optimal ionic strength in the reaction steps of titration of C'4 was 0.09, with the exception of the last step, *i.e.*, between EAC'1,4,2 and C'3.

This modified method was more sensitive and more practical for the titration of C'4 of human serum than the original method. Employing this method, it was found that C'4 titers of normal persons were distributed between 5×10^{12} and 2×10^{13} effective molecules per ml. of serum.

INTRODUCTION

It was reported in the previous paper (Inai *et al.*, 1963) that diminution of complement activity was observed in a patient with chronic myelogenous leukemia, and that this phenomenon was due to the reduction of hydrazine sensitive components (HSC) of complement.

Recently, it was found that in addition to C'4, components of C'3b, C'3c and C'3a were also sensitive to hydrazine (Linscott and Nishioka, 1963). Therefore, in order to investigate the mechanism of diminution of C' activity more precisely, the sera should be examined to see whether C'4 or any of the subcomponents of C'3 decreased. To overcome this problem, a method for titration of these components of complement should be established. However, methods for titration of subcomponents of C'3 have not been reported and it was difficult for us to determine them. On the other hand, Hoffman (Kabat and Mayer, 1961) has reported a method of titration of guinea pig C'4 with intermediate products of immune

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hemolysis as substrates, and with a buffer having an ionic strength of 0.15.

Recently, Wardlaw and Walker (1963) showed the effect of ionic strength on immune hemolysis, and indicated that the maximum hemolytic activity of complement was obtained at an ionic strength of 0.08.

The present investigation was undertaken to improve Hoffman's method by varying the ionic strength of medium for the titration of C'4 in human serum, and to estimate the activity of C'4 in the serum of the above mentioned leukemic patient. Moreover, experimental conditions such as temperature, reaction time at each reaction step, and minimum amount of reagent required for C'4 titration were studied in detail at the optimal ionic strength established from these experiments.

MATERIALS AND METHODS

1. *Complement (C')*

Blood from healthy guinea pigs was obtained aseptically by cardiac puncture without anesthesia. Blood from normal human and the patient was taken aseptically by venous puncture. After standing for about thirty minutes at room temperature the serum was drawn off, centrifuged, and either used on the same day or stored at −20°C before use.

2. *Hemolysin*

Pooled rabbit antiserum against sheep red cell stromata made according to the method of Kabat and Mayer (1961) was employed for all studies.

3. *Diluent*

Stock solution of isotonic NaCl veronal buffer contained 42.5 gm NaCl, 2.875 gm 5,5-diethyl barbituric acid, and 1.875 gm Na-,5 5-diethyl barbiturate in 1,000 ml of distilled water.

Stock solution of isotonic sucrose veronal buffer contained 486 gm sucrose, 2.875 gm 5, 5-diethyl barbituric acid, and 1.875 gm Na-5, 5-diethyl barbiturate in 1,000 ml of distilled water.

For use, both stock solutions were diluted to five fold with distilled water and gelatin (Merck) added to a final concentration of 0.1 per cent. Diluents of various ionic strengths were prepared by mixing diluted NaCl veronal buffer and sucrose veronal buffer. In order to produce a range of ionic strengths from 0.15 to 0.03, both diluted veronal buffers were mixed in the following proportion:

Saline VB	Sucrose VB	Ionic Strength
10	0	0.15
8	2	0.12
6	4	0.09
4	6	0.06
2	8	0.03

Diluents containing calcium (to 0.00015 M) without or with magnesium (to 0.0005 M) were designated as GVB^{+-} and GVB^{++} respectively.

For chelation of Ca^{++} and Mg^{++}, 0.030 M ethylendiamine tetraacetic acid in NaCl-veronal buffer containing neither calcium nor magnesium was used, and designated as EDTA-GVB.

4. *Erythrocytes*

Sheep blood, drawn aseptically, was preserved at 2—5°C with an equal volume of Alsever's solution. On the day of use erythrocytes were washed twice with 0.15 M NaCl and once with

a suitable diluent for each experiment. Sedimented red cells were resuspended in the diluent, and standardized by reading the optical density of a water-lysed aliquot with a Hitachi photoelectric photometer at a wave-length of 530 mμ.

5. *Sensitized sheep erythrocytes (EA)*

Hemolysin was diluted to contain 8 units per ml in the appropriate veronal buffer for each experiment. Diluted hemolysin solution was added to an equal volume of suspension of washed cells containing 10^9 cells per ml, and the mixture was incubated in a water bath at 37°C for 10 minutes for sensitization.

6. *"R" reagent*

R1 was prepared from guinea pig serum by the dialysis method (Kabat and Mayer, 1961). R4 was also prepared from guinea pig serum by the method described in the previous report (Inai *et al.*, 1963).

7. *The first component of complement (C'1)*

C'1 was isolated by the method of Hoffman (Kabat and Mayer, 1961) as follows: A chromatographic column of 60 cm length and 8 cm inside diameter is set up containing 25 gm DEAE-cellulose (Brown Co., Ltd.) in 0.08 M NaCl at pH 7.4. Twenty milliliters of guinea pig serum is diluted with 17.5 ml of distilled water and the mixture is applied to the column. When the serum has just entered the column, 0.08 M NaCl is added, applying pressure with a squeeze bulb to speed up the flow.

The effluent was collected in 10 ml aliquouts and these were checked for their C'1 activities with R1. Effluent containing C'1 activity was collected, the ionic strength adjusted to 0.3 with 3 M NaCl, and calcium added to a final concentration of 0.00015 M. This fraction was stored at −20°C before use.

8. *The second component of complement (C'2)*

C'2 was obtained from guinea pig serum according to the method of Borsos *et al.*, (1961). The fractions obtained were adjusted to an ionic strength of 0.15 and stored at −20°C.

Various lots of C'2 were prepared as above described, and these contained 1.29×10^{11} to 2.66×10^{11} effective molecules of C'2 per ml.

9. *EDTA treated complement (C'-EDTA)*

Guinea pig complement was diluted to 1:12.5 with EDTA-GVB and used as C'3 for the titration of C'4.

10. *The fourth component of complement (C'4)*

C'4 was eluted from the first column employed for the isolation of C'2 as follows: The column was thoroughly washed with 1,000 ml of 0.10 M NaCl followed by 1,000 ml of 0.12 M NaCl. Finally 0.3 M NaCl was applied. The first 50 ml of effluent was discarded. The following effluent was collected in 10 ml aliquots and checked for its C'4 activity with R4. To the active fraction was added calcium at a final concentration of 0.00015 M and the fraction was stored at −20°C. This fraction was found to be accompanied with C'3b, but did not contain C'1.

11. *Titration of complement*

The complement was titrated by the method described in the previous paper (Inai *et al.*, 1963). Wardlow and Walker (1963) titrated guinea pig, human, and rabbit C' in media of various ionic strengths, and found that the highest C' titer was obtained at an ionic strength of 0.08. Using similar experimental conditions we found that the guinea pig C' titer was highest at an ionic strength of 0.09. On the other hand the highest titer of human C' was obtained at 0.15. Although the

causes of this disagreement with Wardlow's results are not clear, C' titration of human sera was performed at an ionic strength of 0.15.

12. *Titration of C'4*

In order to investigate the conditions of each reaction step of titration of C'4 and the influence of ionic strength on the reaction steps, the principle of the titration of C'4 described below was tentatively employed.

To the tubes containing 1.0 ml of diluted C'4 isolated from guinea pig serum or of diluted human serum 0.5 ml of EAC'1 containing 1.5×10^8 cells was added. The mixtures were incubated at 30°C for 20 minutes with occasional agitation. At the end of this incubation period, 1.0 ml of purified C'2 diluted in GVB^{++} was added to each tube, so as to supply about 300 effective molecules per cell. After incubation at 30°C for 30 minutes, each tube received 0.5 ml of whole guinea pig C', diluted 1/12.5 in 0.03 M EDTA-GVB. The tubes were quickly transferred to a water bath at 37°C. After incubation for 60 minutes with occasional agitation, the supernatant fluids were separated by centrifugation at 3,000 rpm for 5 minutes. The hemoglobin content of these supernatants were measured at 414 mμ in a Coleman junior type spectrophotometer.

RESULTS

1. *The effect of ionic strength on titration of C'4*

The effect of ionic strength on titration of C'4 was investigated as follows. Isolated guinea pig C'4 diluted 1/6,000 and fresh human serum diluted 1/200,000 were used as test samples. Buffers having ionic strengths of 0.03, 0.06, 0.074, 0.09, 0.12, or 0.15 were prepared as described above and used for preparation of EA and EAC'1, and for dilution of C'1, C'2 and test samples in each series, with the exception of the last step in which an ionic strength of 0.15 was used in all series.

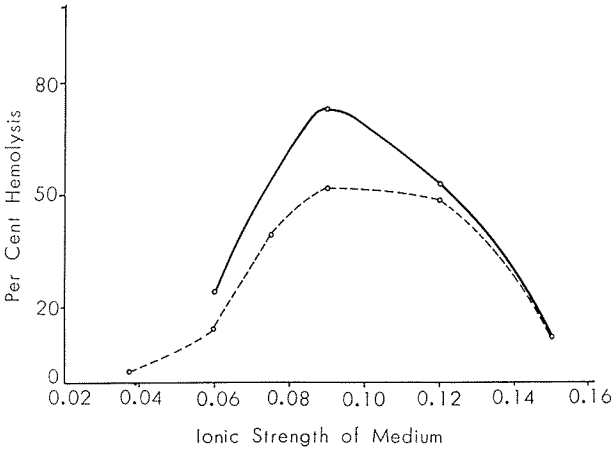

Fig. 1. Effect of Ionic Strength on the Hemolytic Activity of C'4 with Sufficient Amounts of C'1 C'2 and C'-EDTA

o——o Pooled human sera, 1/200,000

o·····o Isolated guinea pig C'4. 1/6,000

As shown in Fig. 1, the degree of lysis in each series was determined, and the percent hemolysis was plotted against the ionic strengths of buffers employed.

It is apparent from this result that hemolytic activity of C'4 is maximum at an ionic strength of 0.09. Therefore, GVB having an ionic strength of 0.09 was used for all following experiments, except for the experiments on the effect of ionic strength on each reaction step. The reason for the use of EDTA-GVB of an ionic strength of 0.15 in the last step is described below.

2. *Preparation of EAC'1*

In order to prepare the most sensitive EAC'1 for titration of C'4, the amount of C'1, optimal ionic strength of the medium and optimal incubation time and temperature in the reaction between EA and C'1 were investigated as follows.

An appropriate amount of C'1 in 20 ml of GVB+− was added to 1.5 ml of EA containing 5×10^8 cells per ml and incubated at 30°C for 15 minutes, and then washed twice with chilled GVB+−. EAC'1 was resuspended in 5.0 ml of GVB+−, and contained 1.5×10^8 cells per ml.

a) *Minimum amount of C'1 required for titration of C'4*

The amount of C'1 was varied and each preparation of EAC'1 was used for estimation of the lytic activity of isolated guinea pig C'4 diluted 1/6,000. Hemolysis in each tube was measured and, as indicated in Fig. 2, the percent hemolysis was plotted against the amount of C'1 used. The minimum amount of C'1 required for titration of C'4 activity was 0.1 ml. In the following experiments 0.2 ml of C'1, a sufficient amount for preparation of EAC'1 was added to 1.5 ml of EA.

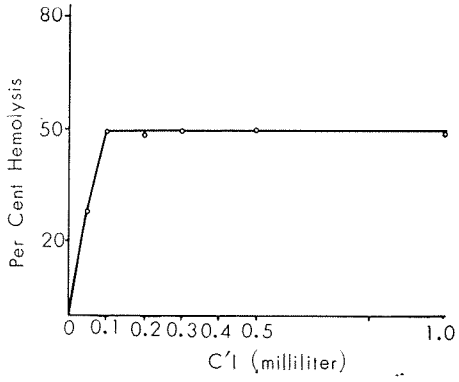

Fig. 2. Amount of C'1 Needed to Prepare the EAC'1 for Obtaining Maximal Activity of C'4

Extent of lysis of each EAC'1 was estimated with a constant amount of C'4 (isolated guinea pig C'4, 1/6,000), C'2 and C'-EDTA.

b) *The incubation time required for preparation of EAC'1*

The incubation time required for preparation of the most sensitive EAC'1 was investigated at 30°C. The time was changed as indicated in Fig. 3. At the end

of incubation, tubes were immediately centrifuged at 1,800 rpm for 10 minutes. The cells were washed twice with chilled GVB+− and resuspended in GVB+−. In order to estimate the sensitivity of EAC'1 thus prepared, the hemolytic activity of guinea pig C'4 diluted 1/6,000 was measured. Maximum lytic activity was obtained with EAC'1 prepared by 15 minutes incubation.

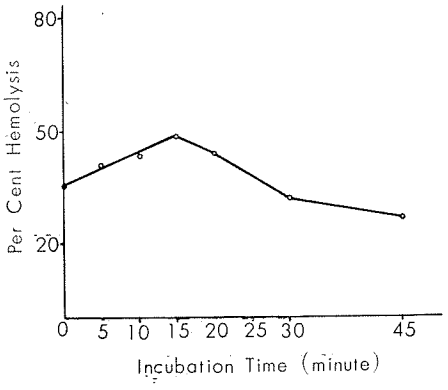

Fig. 3. Reaction Time between EA and C'1 in Preparation of the Most Sensitive EAC'1 for Obtaining Maximal Activity of C'4

EAC'1 was prepared with a constant amount of EA and C'1 (0.2 ml), and with various incubation periods at 30°C. Extent of lysis of each EAC'1 sample was estimated with a constant amount of C'4 (isolated guinea pig C'4, 1/6,000), C'2 and C'-EDTA.

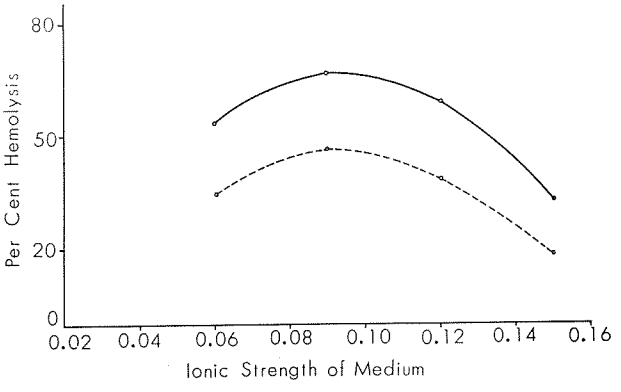

Fig. 4. Effect of Ionic Strength on the Formation of the Most Sensitive EAC'1 for Estimation of C'4 Activity

EAC'1 was prepared with GVB+− of various ionic strengths. Extent of lysis of each EAC'1 sample was estimated with a constant amount of C'4 (isolated guinea pig C'4, 1/6,000 or 1/3,000), C'2 and C'-EDTA.

o——o isolated guinea pig C'4, 1/3,000
o·····o isolated guinea pig C'4, 1/6,000

The same results were obtained in regard to the miniumm amount of C'1 and optimal incubation time to prepare the most sensitive EAC'1 at an incubation temperature of 37°C.

c) *The effect of ionic strength on formation of EAC'1*

EA and EAC'1 were prepared with GVB^{+-} of various ionic strengths and washed twice by the same chilled GVB^{+-}. Finally each EAC'1 was suspended with GVB^{+-} of an ionic strength of 0.09. The hemolytic activity of isolated guinea pig C'4 was estimated by each EAC'1. The ionic strength of the diluent used in the other reaction steps was 0.09, and at the last step EDTA-GVB having an ionic strength of 0.15 was used. The incubation time and temperature of each reaction step of this experiment were as described above. As indicated in Fig. 4, maximum lysis by C'4 was obtained by EAC'1 prepared at an ionic strength of 0.09.

From these results, EAC'1 was prepared as follows: 0.2 ml portion of C'1 in 20 ml of GVB^{+-} at an ionic strength of 0.09 was added to a 1.5 ml portion of EA containing 5×10^8 cells per ml, and incubated at 30°C for 15 minutes. EAC'1 was separated by centrifugation at 2,000 rpm for 10 minutes, and washed twice with the same chilled buffer. Precipitated EAC'1 was suspended in 5.0 ml of the same buffer.

3. *Kinetics of reaction between EAC'1 and C'4*

It is most important for titration of C'4 that all C'4 molecules in the test sample must be attached to EAC'1 and form effective EAC'1,4. Therefore, the kinetics of the reaction between EAC'1 and C'4 were thoroughly investigated.

a) *Effect of incubation time on formation of EAC'1,4*

Isolated guinea pig C'4, diluted to 1/6,000 with GVB^{+-} of an ionic strength 0.09, was used as a test sample. To 1.0 ml of diluted C'4 0.5 ml of EAC'1 was added and the mixture was incubated at 30°C for various periods. The effect of incubation time on formation of EAC'1,4 was investigated by measuring the degree of lysis of EAC'1,4 with C'2 and C'-EDTA. As can be seen in Fig. 5a, maximum hemolysis of EAC'1,4 was obtained at 20 minutes incubation, and lysis of EAC'1,4 was markedly decreased at 60 minutes incubation.

Next, after EAC'1 and C'4 were incubated for a definite time, the reaction mixtures were centrifuged at 2,000 rpm for 10 minutes. Then the precipitated EAC'1,4 cells were washed twice by chilled GVB^{+-}, and resuspended in 1.5 ml of GVB.$^{+-}$ The degree of lysis of these EAC'1,4 cells by C'2 and C'-EDTA was examined. On the other hand, the supernatant of each reaction mixture was analysed for residual C'4 activity. As shown in Fig. 5b, under these experimental conditions the longer incubation time than 20 minutes had no significant effect on the reactivity of EAC'1,4 cells when compared to that of EAC'1,4 obtained after 20 minutes incubation.

Moreover, residual C'4 activity of each supernatant was almost negligible after 20 minutes incubation. From these results, it may be concluded that in these experimental conditions almost all molecules of C'4 in the test sample might be effectively attached to EAC'1.

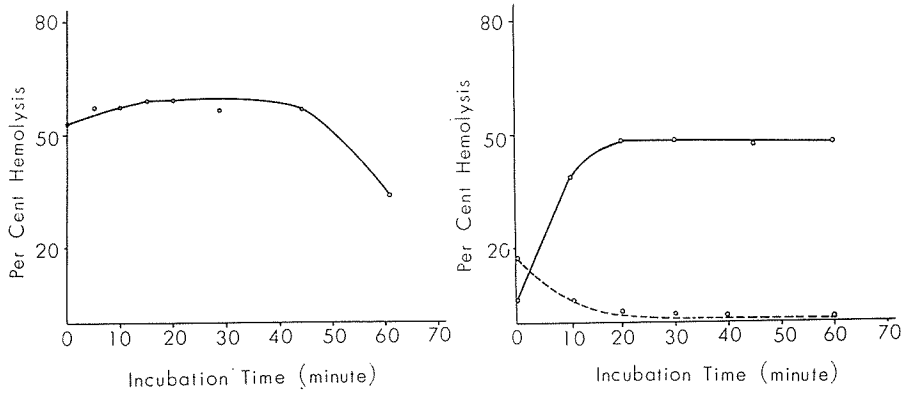

Fig. 5. Kinetics of EAC'1,4 Formation with Constant Amounts of C'1 and C'4 (Isolated Guinea Pig C'4, 1/6,000)

Fig. 5a. Constant amounts of C'2 and C'-EDTA were added without washing EAC'1,4 cells. Extent of lysis of EAC'1,4 was estimated.

Fig. 5b. EAC'1,4 cells were washed at the end of each incubation period. Extent of lysis of each EAC'1,4 sample and the residual C'4 activity remaining in the fluid phase was estimated.

o——o Extent of lysis of each sample of washed EAC'1,4 was estimated with constant amounts of C'2 and C'-EDTA.

o.....o C'4 activity remaining in supernatant. At the end of each incubation period, supernatants were separated by centrifugation of EAC'1,4. To 1.0 ml of each supernatant 0.5 ml of EAC'1 was added, and the extent of lysis of these cells was estimated with constant amounts of C'2 and C'-EDTA.

A similar result was obtained in regard to the optimal incubation time to form the most active EAC'1,4 at an incubation temperature of 37°C.

b) *Effect of ionic strength on the reaction step between EAC'1 and C'4*

The effect of ionic strength on this reaction step was studied as follows:

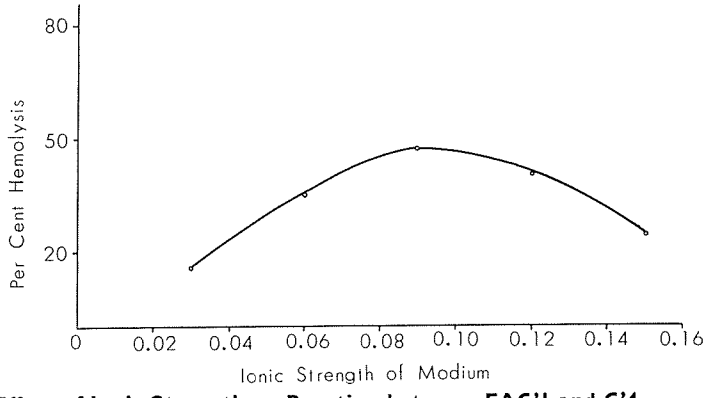

Fig. 6. Effect of Ionic Strength on Reaction between EAC'1 and C'4

Extent of lysis of each EAC'1,4 sample was estimated with constant amounts of C'2 and C'-EDTA. Isolated guinea pig C'4 (1/6,000) was used.

EAC'1, made as described above with an ionic strength 0.09, was divided into five portions and each was centrifuged once and resuspended in GVB+−of ionic strengths of 0.03, 0.06, 0.09, 0.12 and 0.15. To each 0.5 ml portion of the EAC'1, 1.0 ml of isolated guinea pig C'4, diluted to 1/3,000 with GVB+− of corresponding ionic strengths, was added. After incubation for 20 minutes at 30°C, each EAC'1,4 was centrifuged, and all cell residues were resuspended in 1.5 ml of GVB+− of an ionic strength 0.09.

The hemolytic activity of each EAC'1,4 was estimated by adding sufficient amounts of C'2, diluted with GVB++ of an ionic strength 0.09, and C'-EDTA. As shown in Fig. 6, the optimal ionic strength at this step for yielding maximal activity of C'4 was 0.09.

4. *Kinetics of reaction between EAC'1,4 and C'2*

In studies on the kinetics of the reaction between EAC'1.4 and C'2 the amount of C'2 required for the formation of EAC'1,4,2 and the effect of the ionic strength of the medium on this step were examined, using the same incubation time and temperature as reported by Borsos *et al.* (1961).

a) *Minimum amount of C'2 required for the formation of EAC'1,4,2 from EAC'1,4*

An Erlenmeyer flask, placed in a water bath at 30°C, was charged with 5.0 ml of EAC'1 (1.5×10^8 cells per ml) prepared as described above, and with 10 ml of isolated guinea pig C'4 diluted to 1/6,000 in GVB+−. After 20 minutes incubation, 1.5 ml of the suspension was immediately pipetted into tubes containing varying numbers of effective molecules of C'2 in 1.0 ml of GVB++. After incubation at 30°C for 30 minutes, each tube received 0.5 ml of C'-EDTA, as described above.

As shown in Fig. 7, at 100 effective molecules per cell of C'2 the maximum hemolysis of EAC'1,4 could be seen. Therefore, it was considered that in the titration of C'4, 300 effective molecules per cell of C'2 was sufficient.

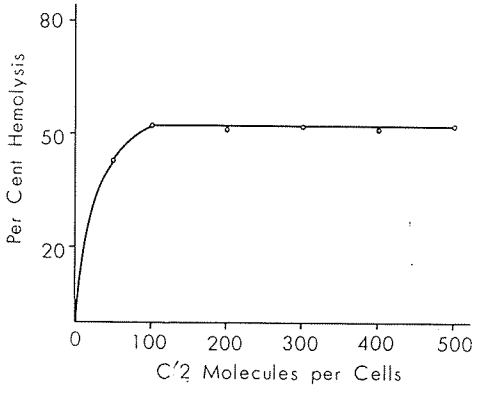

Fig. 7. Hemolysis of EAC'1,4 with Varying Amounts of C'2 and a Constant Amount of C'-EDTA

Isolated guinea pig C'4 (1/6,000) was used for preparation of EAC'1,4.

In the reaction step between EAC'1,4 and C'2, incubation at 30°C for 30 minutes was employed, according to the method of Borsos *et al.* (1961).

b) *Effect of ionic strength on the reaction step between EAC'1,4 and C'2*

The effect of ionic strength on this step was investigated as follows. An Erlenmeyer flask was charged with 10.0 ml of EAC'1, 1.5×10^8 cells per ml, and with 20.0 ml of isolated guinea pig C'4, diluted to 1/3,000 with GVB+− of an ionic strength 0.09. After incubation for 20 minutes at 30°C, the EAC'1,4 was divided into 5 portions and each was centrifuged once and resuspended in 6 ml of GVB+− of ionic strength 0.03, 0.06, 0.09, 0.12 and 0.15.

To each 1.5 ml portion of the EAC'1,4, 1.0 ml of C'2 which was diluted with GVB++ of corresponding ionic strength so as to supply 300 effective molecules per cell, was added. After incubation for 30 minutes at 30°C, each sample of EAC'1,4,2 was centrifuged once, and all cell pellets were resuspended in 2.5 ml of GVB++ of ionic strength 0.09. The hemolytic activity of each EAC'1,4,2 was estimated by the addition of C'-EDTA.

As shown in Fig. 8, the optimal ionic strength of this reaction step to yield maximal activity of C'4 was 0.06.

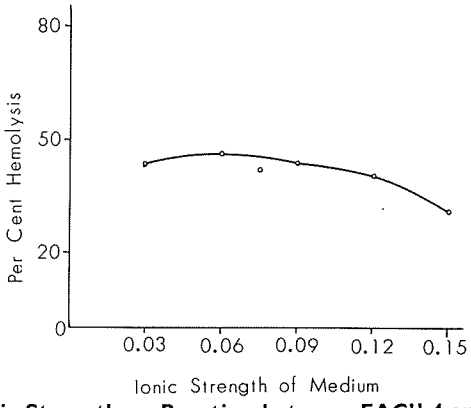

Fig. 8. Effect of Ionic Strength on Reaction between EAC'1,4 and C'2
Extent of lysis of each EAC'1,4,2 was estimated with a constant amount of C'-EDTA. Isolated guinea pig C'4 1/6,000 was used for preparation of EAC'1,4.

5. *Kinetic analysis of the conversion of EAC'1,4,2 to E* by C'-EDTA*

An Erlemeyer flask contained 10 ml of EAC'1 and 20 ml of isolated guinea pig C'4 diluted to 1/6,000 in GVB+− was incubated at 30°C for 20 minutes. Next, 20 ml of C'2, which was diluted with GVB++ so as to supply 300 effective molecules per cell, was added. After incubation at 30°C for 30 minutes, the suspension was divided into two portions. The one portion was transferred to a flask in a water bath at 37°C and the other to a flask in a bath at 30°C. To each flask, 5 ml of guinea pig C' diluted to 1/12.5 with EDTA-GVB was quickly added, and then at suitable intervals, 3.0 ml samples were withdrawn from each flask, immediately

centrifuged, and the optical density of the supernatant fluid was read at 414 mμ in a Coleman junior type spectrophotometer.

As shown in Fig. 9, the degree of lysis, reached its maximum after 30 minutes at 37°C and after 60 minutes at 30°C, but the degree of maximal lysis was almost the same in both cases. Therefore, at this step samples were incubated at 37°C for 60 minutes.

EDTA-GVB was made with saline veronal buffer of an ionic strength of 0.15, because variation of the ionic strength of C'-EDTA had no effect on the lysis of EAC'1,4,2.

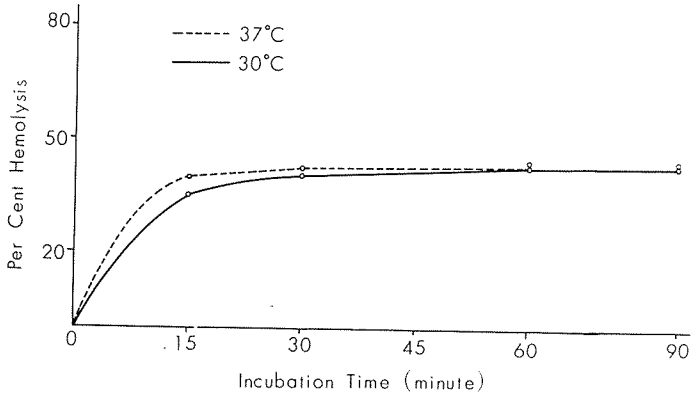

Fig. 9. Effect of Incubation Time and Temperature on the Extent of Lysis of EAC'1,4,2 with a Constant Amount of C'-EDTA

Isolated guinea pig C'4 1/6,000 was used for preparation of EAC'1,4,2.

6. *Titration of C'4 in whole human serum*

An ionic strength of 0.09 was employed throughout this titration system, including washing and sensitization of erythrocytes. But at the last step, the ionic strength of the EDTA-GVB employed in the dilution of C' was 0.15. All samples were accurately diluted and the incubation time and temperature in each step was decided by the results of above experiments. Cell and EAC'1 controls were set up, and a complete lysis control was made with distilled water. After the unlysed cells in each tube had been separated by centrifugation, optical densities of the supernatant fluids were read at 414 mμ in a Coleman junior type spectrophotometer.

The protocol of a sample experiment is shown in Table 1.

For calculation of the C'4 titer, $-In$ (1-y) was calculated from the per cent hemolysis (y), and plotted against the reciprocal of the dilution of whole serum according to the one hit theory (Borsos and Mayer, 1961). This should yield a straight line. The dilution of whole serum at which $-In$ (1-y) = unity was then read from the graph. The reciprocal of this value was multiplied by the number of cells (7.5×10^7) to obtain the effective C'4 molecules per ml. The C'4 activities of 33 normal human sera, of 17 males and 16 females, were titrated by the method described above.

Table I. The Protocol of the Titration of C'4 in Human Serum

	Tube No.							
	1	2	3	4	5	6	7	8
EAC'1 ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
1/serum dil. 1.0 ml	5×10^4	10×10^4	20×10^4	40×10^4	80×10^4	—	—	—
Buffer (GVB^{+-}) ml	—	—	—	—	—	1.0	2.5	2.5 *
Incubated for 15 min. at 30°C								
C'2 (diluted in GVB^{++}) ml	1.0	1.0	1.0	1.0	1.0	1.0	—	—
Incubated for 30 min. at 30°C								
C'-EDTA 1:12.5 ml	0.5	0.5	0.5	0.5	0.5	0.5	—	—
Incubated for 60 min. at 37°C								
Centrifuged. O.D. of supernatant fluid determined								
O.D. 414	0.680	0.560	0.425	0.315	0.220	0.065	0.003	0.750
corrected O.D.	0.615	0.495	0.360	0.250	0.155			0.747
y	0.825	0.663	0.482	0.335	0.208			
1 — y	0.175	0.337	0.518	0.665	0.792			
—ln(1—y)	1.74	1.09	0.658	0.407	0.237			

$11\times10^4\times7.5\times10^7=8.2\times10^{12}$ Effective C'4 molecules/ml * H_2O

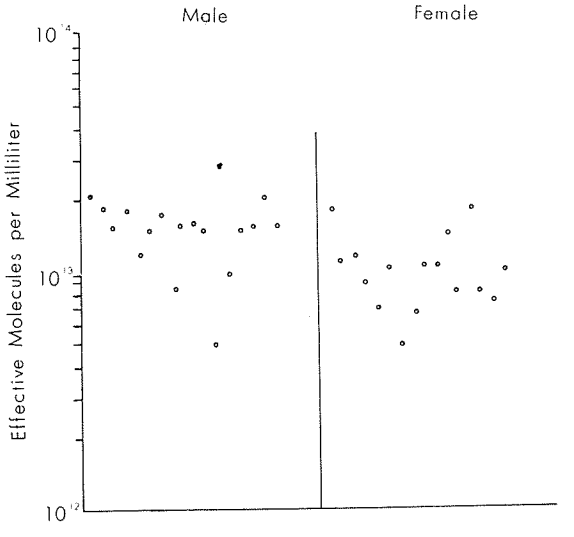

Fig. 10. Distribution of C'4 Titers of 33 Normal Human Sera

As seen in Fig. 10, C'4 titers were distributed from 5×10^{12} to 2×10^{13} effective molecules per ml, and no difference was observed between males and females.

7. *C'4 titers of a patient with chronic myelogenous leukemia*

As reported in the previous paper (Inai *et al.*, 1963), during the clinical course of a patient with chronic myelogenous leukemia, a marked reduction of C' activity of the sera was observed. Some of these sera had been stored at −20°C.

We attempted the titration of C'4 of these stored sera, and at the same, C' activities were also titrated again, since reduction of C' and C'4 activities might have occurred during the period of storage.

Table 2. Comparison between C', H.S.C. and C'4 Titers of the Patient with Chronic Myelogenous Leukemia

Date	C'(C'H50)[1]	HSC(u)	C'(C'H50)[2]	C'4 mol/ml
1/II	3.2	15	2.0	7.5×10^{10}
7/II	44.5	235	17.7	7.5×10^{11}
29/III	49.0	910	29.0	7.5×10^{10}
13/V	55.6	3750	—	2.6×10^{12}
18/VI	30.8	500	27.8	9.5×10^{9}
26/VI	28.1	333	21.2	1.9×10^{10}
10/VII	59.8	5000	38.5	2.1×10^{12}

1) Determined before storage.
2) Determined after storage at—20°C. C'4 was titrated at the same time.

As shown in Table 2, C'4 activities of the sera which had revealed a low C' titer were also found to be below the normal range; for example, those of the sera of Feb. 1, Feb. 7, March 29, June 18 and June 26. The serum of Feb. 1, especially when titrated for C'4 activity, showed no hemolysis even when 1.0 ml of this serum diluted to 1/1,000 was used. As is seen in this Table, on the second titration of these sera some reduction of C' activities was observed, suggesting the partial inactivation of the components of complement during storage. However, it may be reasonable to assume that the low titer of C'4 in these sera might represent the intrinsic properties of these sera rather than the partial inactivation of C'4 due to storage, because the C'4 titer of the serum of May 13 was found to be much higher than that of June 18 or June 26.

DISCUSSION

The titer of C'4 is usually estimated with R4, the classical serum reagent for C'4 activity. Recently, Müller-Eberhard *et al.* (1961) and Linscott and Nishioka (1963) have shown that C'3c, C'3b and C'3a are also sensitive to hydrazine. It has also been shown that the activity of C'4 can be estimated with intermediate products of immune hemolysis (Kabat and Mayer, 1961). On the other hand, an

ionic strength of 0.08 was found to be optimal for the hemolytic activity of complement (Wardlow and Walker, 1963) and for formation and lysis of EAC'1,4,2 (Walker and Wardlow, 1963).

In order to investigate the C'4 activities of serum of the patient with chronic myelogenous leukemia who revealed markedly low titers of C' and HSC, the authors have reexamined Hoffman's method in detail. C'1, isolated by the method of Hoffman (Kabat and Mayer, 1961), was contaminated with C'2, subcomponents of C'3, and with a very small amount of C'4. The effect of contamination of this C'1 with a very small amount of C'4 was negligible, as shown in the control tube (see Table 1). The contaminating C'2 and subcomponents of C'3 had no effect on titration of C'4, since they were washed off after sensitization of EA with C'1 in GVB+−. C'2 isolated by the method of Borsos *et al.* (1961) contains C'3d (Linscott and Nishioka, 1963), but its effect was negligible, since sufficient C'-EDTA was added at the last step. The effect of C'3b contamination on isolated guinea pig C'4 was also negligible.

To yield maximal activity of C'4, an ionic strength of 0.09 was found to be optimal in the reaction step between EA and C'1, and between EAC'1 and C'4, or whole serum to be tested. And an ionic strength of 0.06 was found to be optimal in the reaction step between EAC'1,4 and C'2. However, in the last step, the reaction between EAC'1,4,2 and C'-EDTA, was unaffected by variation of the ionic strength from 0.03 to 0.15. As to the incubation time and temperature of each step, the results were almost identical to those of Hoffman, despite the lower ionic strength used in our experiment.

Recently, isolation and purification of the fourth component of human complement was reported by Müller-Eberhard and Biro (1963). They concluded that purification of the fourth component of human complement resulted in the isolation of β 1E-globulin, and this represents the fourth component of human complement. But, it was reported that approximately 250 molecules per cell of purified C'4 were needed to achieve 63% lysis of cells and only a certain percentage of these molecules combined with the cell. However, as described above, in our experimental conditions all of effective molecules of C'4 in the test sample may be attached to EAC'1, and it is difficult to assume that effective molecules remained in the fluid phase.

The C'4 titer of normal human whole serum expressed as the effective number of molecules has not previously been reported, although Hoffman reported that the C'4 titer of guinea pig whole serum was 3 to 4 $\times$ 10^{12} effective molecules per ml. Employing the above method, the C'4 titers of 33 normal human sera were found to be distributed from 5×10^{12} to 2×10^{13} effective molecules per ml.

A possible correlation between the HSC titers and C'4 titers of the patient with chronic myelogenous leukemia was recognized. However, the assumption that the low HSC titers of this patient were due to the mere depletion of C'4 was not confirmed in this experiment, because the hydrazine sensitive subcomponents of C'3 were not investigated.

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