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STUDIES ON THE C'3d OF GUINEA PIG COMPLEMENT[1]

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SUMMARY Kinetic studies were made on S* formation from SAC'1, 4, 2, 3c, 3b, 3e, 3f, 3a (a-site) and isolated C'3d. S* was shown not to decay unless it was lyzed. The method of titration of C'3d activity was examined and it was clearly shown that C'3d can be depleted by a-sites resulting in the conversion of these sites to S*'s. From these results a method was devised for titration of C'3d.

INTRODUCTION

C'3d was discovered by NISHIOKA and LINSCOTT (1963) as the last reacting complement component in immune hemolysis. They reported that this component was not depleted from guinea pig serum by immune precipitate, and thought that it might be enzymic in nature. However, their conclusion was based on an inadequate method for titration of the components.

We studied the isolation and purification of the components of the guinea pig C'3 group. Using these preparations we prepared EAC'1, 4,2,3c,3b,3e,3f,3a (a-cells), and studied the formation and lysis of S* kinetically. We also showed that C'3d can readily be removed from the reaction medium by a-sites, resulting in the formation of S*. From this fact, a method was devised for titration of C'3d.

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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 (1961). 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 buffered solution of low ionic strength was made by mixing VB-saline and VB-sucrose in appropriate proportions and the ionic strength was checked by measuring its conductivity. A mixture of equal volumes of VB-saline and VB-sucrose (VBSS) [pH 7.3-7.5, μ=0.076] was also used. VB-saline containing 0.005 M ethylene-diamine tetraacetate (0.005 M EDTA-VB-saline) was prepared by mixing 1 part of 0.01 M trisodium ethylene-diamine tetraacetate (pH 7.5 to 7.7) with 19 parts of VB-saline prepared without added Mg^{++} or Ca^{++}. Isotonic EDTA solution containing 0.1% gelatin (0.09 M EDTA) was prepared according to the description of FRANK *et al.* (1964). Isotonic veronal buffered potassium chloride solution con-

taining 0.1% gelatin (VB-KCl) was prepared like VB-saline except that it contained 1.19% (w/v) KCl instead of NaCl.

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

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

3. *Sensitized erythrocytes (EA)*

Sheep erythrocytes were washed twice with isotonic saline, and then three times with 0.005 M EDTA-VB-saline. The suspension was adjusted to 1.0×10^9 cells/ml and mixed with an equal volume of antiserum diluted in the same buffer to give the optimal sensitization. After shaking at 37°C for 30 min and then at 0°C for more than one hour, the cells were washed once with 0.005 M EDTA-VB-saline and three times with VB-saline or VBSS.

4. *Separation and isolation of C'3d and the reagent containing C'4, C'2, C'3c, C'3b, C'3e and C'3f (4-f reagent)*

These reagents were obtained according to a modification of the method of NELSON *et al.* (1966). Briefly, 20 ml of guinea pig serum were diluted slowly with 55 ml of ice-cold distilled water and adjusted to pH 7.5 with 0.04 N HCl using a magnetic stirrer. After standing at 0°C for 30 min the serum was centrifuged in the cold. The precipitate was separated and used as a source of C'1 in other experiments. The supernatant was mixed with 1/9 volume of 0.2 M acetate buffer, pH 5.0, and adjusted to pH 5.0 with 0.1 N acetic acid. It was applied on a column of CM-cellulose (3 × 30 cm) equilibrated with 0.04 M NaCl in 0.02 M acetate buffer, pH 5.0. The column was then eluted with 1,000 ml of a linear gradient from 0.12 M to 0.15 M NaCl in 0.02 M acetate buffer, pH 5.0. Ten ml fractions were collected in tubes, containing sufficient 0.2 M tris (hydroxymethyl) aminomethane to adjust the pH to neutrality, using an automatic fraction collector. The column was then eluted with 500 ml of 0.15 M NaCl in 0.02 M acetate buffer, pH 5.0. The resulting fractions were titrated for C'2, C'4, C'3f, C'3e, C'3b and C'3c. Fractions containing these components were pooled and concentrated by ultra-filtration in the cold if necessary. Then sucrose was added to give isotonicity and then 1.0 per cent gelatin was added. The material (4-f reagent) was stored in a deep freezer at -20°C until use.

Then the column was washed with about 3 liter of 0.17 M NaCl in 0.02 M acetate buffer, pH 5.0, and then C'3a and C'3d were eluted together with 0.4 M NaCl. Fractions were pooled, adjusted to pH 7.5 with 0.2 M tris (hydroxymethyl) aminomethane, and to ionic strength, $\mu=0.05$, with distilled water. This fraction was applied to another column of DEAE-cellulose (3 × 30 cm) equilibrated with NaCl in 0.005 M phosphate buffer, pH 7.5, of $\mu=0.05$. The nonadsorbed fraction contained C'3a and this was purified by rechromatography on a CM-cellulose column. The C'3d remaining on the DEAE-cellulose column was eluted with an increasing concentration of NaCl in 0.005 M phosphate buffer, pH 7.5. The C'3a and C'3d thus obtained were rechromatographed on DEAE- or CM-cellulose columns.

Alternatively, C'3d could be obtained by Sephadex G-200 column chromatography of whole serum as a slow-moving portion and purified further by rechromatography on DEAE- or CM-cellulose columns. C'3a could be obtained by chromatography of whole serum on DEAE-cellulose or DEAE-sephadex with an increasing concentration of NaCl and purified by further chromatography on DEAE- or CM-cellulose.

5. *Partially purified C'2 preparations*

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

6. *Intermediate cells of immune hemolysis*

EAC'1,4 cells were prepared as described by INOUE and NELSON (1965, 1966).

EAC'1, 4, 2, 3c, 3b, 3e, 3f (f-cells) were made by incubating EAC'1, 4, 2 with an appropriate dilution of 4-f reagent at a final cell concentration of 1 to 5×10^8 cells/ml in VBSS at 30°C for 20 to 25 minutes. The cells were washed several times with VBSS and could be stored in the same buffer at 0°C for at least 2 weeks.

EAC'1, 4, 2, 3c, 3b, 3e, 3f, 3a (a-cells) were prepared by incubating washed f-cells with an appropriate dilution of C'3a in VBSS at 30°C for 15 to 30 minutes.

RESULTS

1. *Generation of S* from SAC'1,4,2,3c,3b,3e,*

3f,3a (a-site) and C'3d and its lysis

Expt. 100565

The suspension of a-cells at a concentration of 1.0×10^8 cells/ml in 0.005 M EDTA-VB-saline, was preincubated at 30°C, and then mixed with an equal volume of 1/1,100 C'3d (preparation #5) in the same buffer at 30°C, at time 0 in an Erlenmeyer flask immersed in a water bath of 30°C. The flask was shaken continuously to keep the cell suspension homogeneous. At intervals (time), 1.0 ml aliquots were taken into tubes containing 10 ml of cold 0.09 M EDTA. The contents were mixed well and centrifuged in the cold. The supernatant (Sup. 1) was removed, and the tubes were drained and wiped with filter paper. The cells were resuspended in 3.0 ml of 0.005 M EDTA-VB-saline, and incubated again at 30°C for 180 min with constant shaking. Then 8.0 ml of cold 0.005 M EDTA-VB-saline was added and tubes were centrifuged. The optical densities of the supernatant (Sup. 2) and Sup. 1 were read at 413 mμ. From the reaction flask, 1.0 ml aliquot was taken into a tube containing 10 ml of cold distilled water and used as the control for total lysis. The control system containing a-cells only was treated similarly. The optical densities of Sup. 1 and Sup. 2 of this control system showed less than 2 per cent of the total lysis of the control.

The ratios of lysis in Sup. 1 and 2 to the total lysis of the control were calculated as y_1 and y_2, respectively. Then $z_1=-\ln(1-y_1)$ represents the average number of sites on a cell which caused cell lysis in time t, and $z=-\ln(1-y_1-y_2)$ is the average number of $\vec{S}^*$, *i.e.*, the total number of S* which were formed by time t. The difference, $-\ln(1-y_1-y_2)+\ln(1-y_1)$ represents the average number of S* present on a cell at time t.

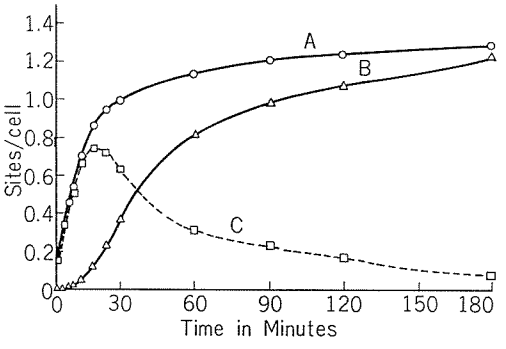

FIGURE 1 The formation of S* from a-site and C'3d and its lysis.

Curve A: $\vec{S}^*$, Curve B: ghost, Curve C: S*

As shown in Fig. 1, the generation of S* started without any lag period, while the lysis of cells started after a certain lag period.

Expt. 011366

Flasks containing 19 volumes of various concentrations of C'3 d (Preparation #7) (A: 1/190, B: 1/950, C: 1/2,800, and D: 1/4,750) in 0.005 M EDTA-VB-saline or this buffer only (0) were suspended in water baths at 0°C, 30°C and 37°C. Portions of a sample of a-cells at a concentration of 1.0×10^9 cells/ml were put into 3 flasks and each was equilibrated at one of the above temperatures. At time 0, one volume of cell suspension was pipetted into a flask equilibrated at the same temperature. The procedures thereafter were the same as for Expt. 100565. The second step of incubation (lysis of the S* remaining) was carried out at 30°C for 180 min. Fig. 2 shows the $\vec{S}^*$ curves obtained in this experiment.

As shown in this figure, the generation of S*

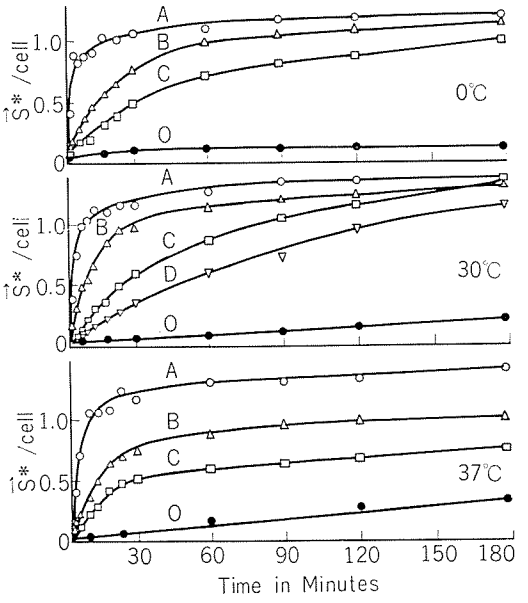

FIGURE 2 Kinetics of S* formation at various temperatures.

The concentrations of C'3d (preparation #7) were 1/200 to A, 1/1,000 to 5, 1/3,000 to C and 1/5,000, and the control 0 contained no C'3d.

proceeded rapidly even at 0°C, while lysis proceeded slowly at this temperature (not shown here). With lower concentrations of C'3d, the generation of S* at 0°C and 30°C continued and reached nearly the value obtained with higher C'3d concentrations after a long incubation period. At 37°C, however, the generation with lower C'3d concentrations stopped relatively earlier and reached a different plateau value. The difference in the modes of generation of S* might result from differences in the decay rates of a-sites at different temperatures.

In fact the half life of a-sites was observed to be about 200 min at 37°C and ca. 800 min at 30°C (MORI and INOUE unpublished). At 37°C, therefore, rapid decay of a-sites might cause a different end-value of $\vec{S}$* formed. On the other hand, C'3d itself was stable and its activity was retained even after 24 hours incubation at 30°C in 0.005 M EDTA-VB-saline.

2. *Lysis of E* and possible decay of S**

Expt. 061466

A suspension of a-cells which contained about 1.0 a-site/cell in 0.09 M EDTA was mixed with a large excess of C'3d at 0°C and shaken for 6 min. After centrifuging and washing three times with cold 0.09 M EDTA, the cells were resuspended in the same diluent and adjusted to a concentration of 2.0 $\times 10^9$ cells/ml. The resulting sample of E*-cells was divided into 3 parts. At zero time one part was pipetted into 19 parts of 0.09 M EDTA prewarmed at 30°C, the second part was mixed with 19 parts of VB-saline, and the third part was put into an empty flask suspended in a water bath at 30°C.

All the reaction mixtures were incubated at 30°C with constant shaking. Then 31 min after the start of the reaction, 1 part of the third sample was pipetted into another flask containing 19 parts of VB-saline. At intervals, 0.5 ml of each reaction mixture was taken into 10.0 ml of cold 0.09 M EDTA, mixed well and centrifuged immediately in the cold. The optical density of the supernatant was measured at 413 mμ. The precipitated cells from the first sample were resuspended in 3.0 ml of 0.005 M EDTA-VB-saline and incubated further at 30°C for 180 min. Then 7.5 ml of cold 0.09 M EDTA were added and the mixture was centrifuged. The optical density of the supernatant was read at 413 mμ. From the readings for the first and second supernatants $\vec{S}$* at time t was calculated as in Expt. 100565, and is shown in curve #1′ in Fig. 3.

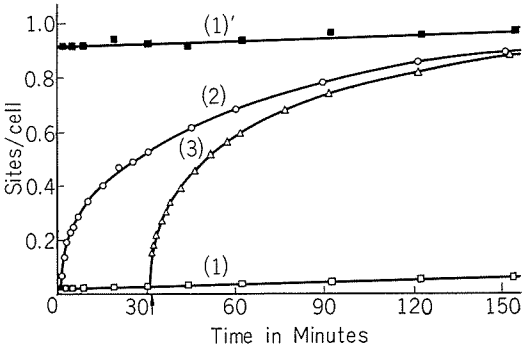

FIGURE 3 Lysis and possible decay of S*.

As shown in Fig. 3, the lysis of E* was inhibited by 0.09 M EDTA (Curve #1) (FRANK *et al.*, 1964) but E* was lysed after a short lag period in 0.005 M EDTA-VB-saline (Curve #2).

On the other hand, in the third reaction system there was no lag period and a very steep rise in the hemolytic curve was seen (Curve #3). These facts may reflect the temperature-dependent step of the E* precursor described by FRANK *et al.* (1965). Curve #1′ shows clearly that S* did not decay, at least at 30°C.

3. *Ionic strength and generation of S**

Many reports have appeared on the relationship between the ionic strength of the supporting medium and the hemolytic activity of complement or its components (RAPP and BORSOS, 1963, BORSOS and RAPP 1963, INOUE and NELSON, 1965, 1966). We also investigated the influence of ionic strength on the formation and lysis of S*. Diluents of various ionic strength were prepared by mixing VB-saline and VB-sucrose as described in Materials and Methods.

Expt. 101766

Samples of 0.1 ml of a-cell suspension at a con-

centration of 1.0×10^9 cells/ml in VBSS were prewarmed at 30°C, and mixed with 4.0 ml samples of C'3d diluted with each of the diluents at 30°C. The reaction mixtures were incubated at 30°C for 12 minutes with constant shaking. After incubation, 5.0 ml of the same diluent as before at 0°C was added and tubes were centrifuged in the cold. The optical density of the supernatant was measured at 413 mμ. The sedimented cells were resuspended in 3.0 ml of 0.005 M EDTA-VB-saline and incubated at 37°C for 90 min. The $\vec{S}$* formed was calculated as described before. For comparison, a tube containing 0.09 M EDTA instead of VB-sucrose-saline mixture was included.

As shown in Fig. 4, at ionic strengths above 0.05 the amounts of S* formed were about the same, while at below 0.05 formation was markedly less. In 0.09 M EDTA, S* formation was moderately reduced.

4. *Ionic strength and lysis of E**

Expt. 053066

E* cells were made by reacting a-cells and C'3d in 0.09 M EDTA at 0°C. After centrifuging and washing them several times with the same diluent, the cells were incubated at 30°C for 60 min in 0.09 M EDTA to allow the temperature-dependent step of E*. The cells were then resuspended in 0.09 M EDTA at a concentration of 1.0×10^9 cells/ml.

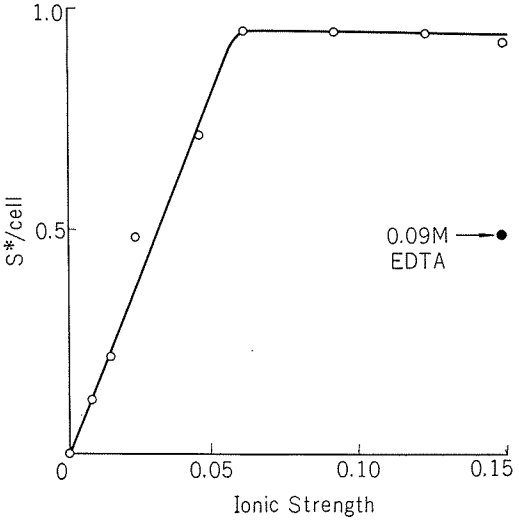

FIGURE 4 Ionic strength and formation of S*.

Samples of 0.1 ml of this suspension were pipetted into 4.0 ml of diluents of various ionic strength, prewarmed at 30°C. After incubation at 30°C for 20 min, each reaction mixture received 5.0 ml of cold 0.09 M EDTA, and was then centrifuged. The optical densities of the supernatants were measured at 413 mμ (Curve A). The sedimented cells were resuspended in 3.0 ml of 0.005 M EDTA-VB-saline and incubated further at 30°C for 120 min. After mixing with 6.0 ml of cold 0.09 M EDTA, the cells were centrifuged. The number of S*, which had been lyzed during the first 20 min of incubation was calculated from the first supernatant. The numbers of S* lyzed plus those which remained at the end of the incubation period were calculated from both the first and second supernatants (Curve B).

As shown in Fig. 5, lysis of S* was accelerated at higher ionic strength. However, the sum of lyzed and remaining S* sites at the end of the incubation period was the same at all ionic strengths tested, which showed that the poor lysis observed at lower ionic strength was not due to destruction of S* but only to

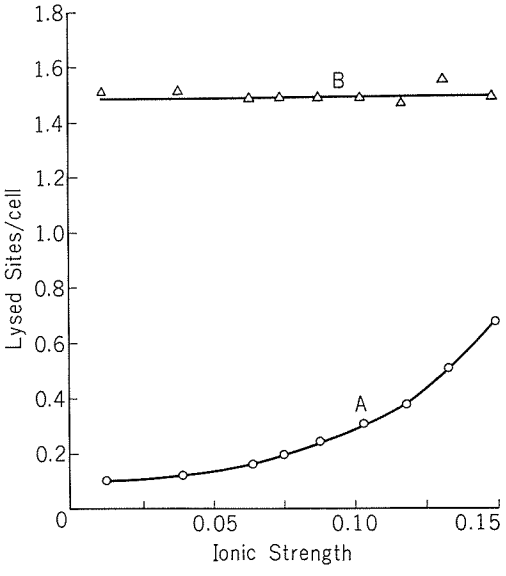

FIGURE 5 Ionic strength and lysis of S* (in VB-saline).

Curve A represents the amount of lysis of S* during 20 min incubation at 30°C, curve B represents the total amount of lysed and remaining S* at the end of the incubation period.

delay in its lysis.

The acceleration of lysis of E* observed in media of higher ionicity, however, might result not from a quantitative effect of a cation but from a qualitative influence of the sodium ion used in the experiment upon the equilibrium between intracellular potassium and extracellular sodium ions. However, this possibility was excluded by the following experiment using VB-KCl instead of VB-saline (Fig. 6, Expt. 063066). In this case, the lysis of E* was accelerated at higher ionic strength regardless of the kind of cation present.

5. *Analysis of the early stage of S* formation*

As shown in Fig. 2, at 0°C and 30°C especially, the numbers of $\vec{S}$* formed after a long incubation period seem to depend only on the number of a-sites on the cells at the start of the reaction and not on the concentration of C'3d used.

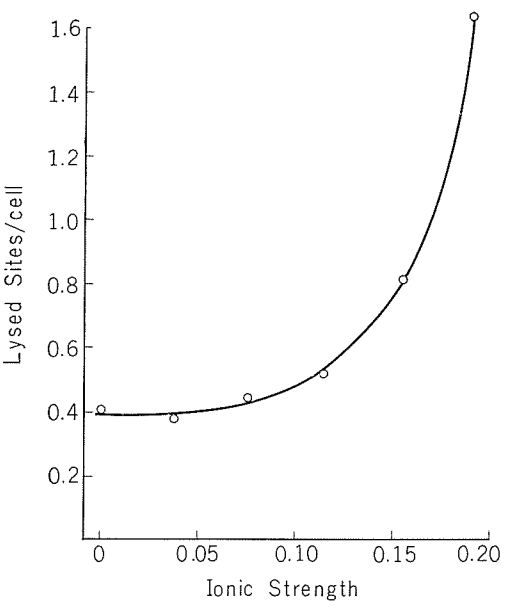

FIGURE 6 Ionic strength and lysis of S* (in VB-KCl).

The values represent the numbers of lyzed S* after incubation at 30°C for 7 min.

If this is the case, the concentration of C'3d cannot be determined by measuring the S* formed after a long incubation period. Therefore, analysis of the early step of S* formation was attempted.

Expt. 040866

To 19 volumes of C'3d (preparation #7, 1/950) diluted in 0.005 M EDTA-VB-saline at 30°C were added one volume of a-cells at concentrations of 1.0×10^9, 1.5×10^9 and 2.0×10^9 cells/ml in the same buffer, at 30°C. At short intervals, 1.0 ml aliquots were taken from each reaction mixture into tubes each containing 10.0 ml of cold 0.09 M EDTA. The tubes were mixed well and centrifuged immediately. The optical density of the supernatant was measured at 413 mμ and contained no detectable oxyhemoglobin. The sedimented cells were resuspended in 3.0 ml of EDTA-VB-saline and incubated at 37°C for 90 min with constant shaking. After this second incubation period, the cells were centrifuged and the optical density of the supernatant was measured at 413 mμ for calculating the S* formed during the first reaction period.

As shown in Fig. 7, A, S* formation in the early stage of the reaction was linear at each a-cell concentration. When the reciprocals of the initial velocities of the reactions were plotted against the reciprocals of the cell concentrations (Lineweaver-Burk's plot), a straight line which passed near the origin was obtained, as shown in Fig. 7, B.

6. *The relationship between the initial velocity of S* formation and the concentration of C'3d using a constant concentration of a-cells*

Expt. 071766

Four batches of a-cells were prepared. The average numbers of a-sites on the cells of each batch were estimated by lyzing them with a large excess of C'3d, and were represented as *Za*. For each cell preparation, seven tubes were set up, five tubes containing 1.0 ml of varying concentrations of C'3d (preparation #8) in 0.005 M EDTA-VB-saline and two tubes containing the same diluent without C'3d (the concentration of C'3d was twice that for Fig. 8). The tubes were warmed to 30°C in a water bath. To each tube 1.0 ml of a-cells at a concentration of 2.0×10^8 cells/ml, at 30°C, were added. Each tube was incubated at 30°C with mechanical shaking for exactly six minutes. After incubation, 10 ml of

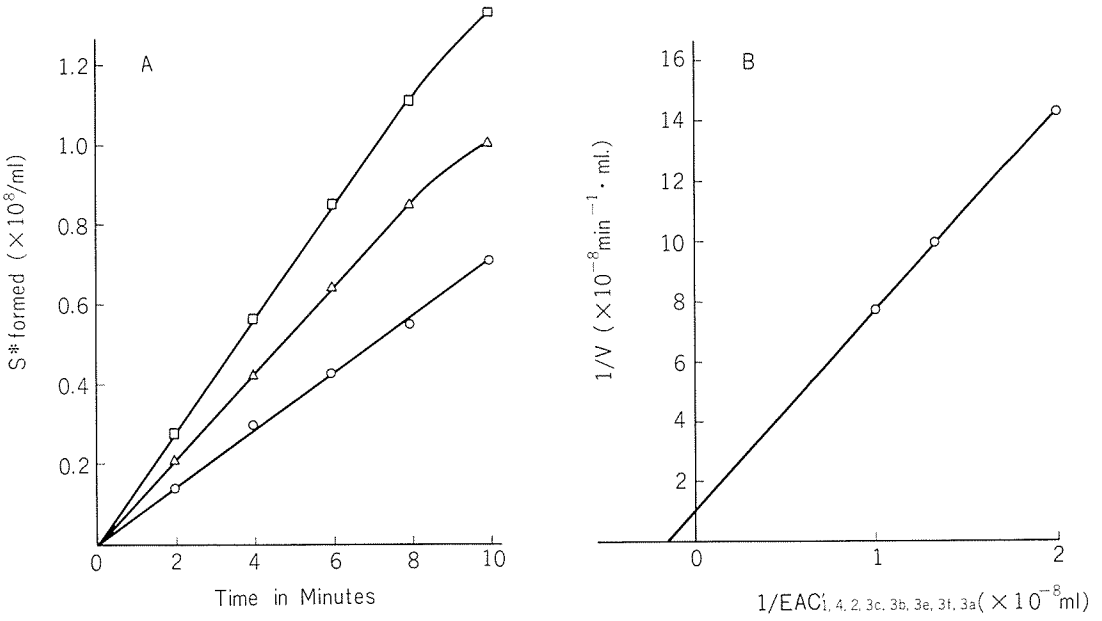

FIGURE 7 Relationship between the initial velocity of S* formation and the concentration of a-cells using a constant concentration of C'3d.
□——□: a-cells at concentration of 10.0×10^{7} cells/ml, △——△: a-cells at concentration of 7.5×10^{7} cells/ml, ○——○: a-cells at concentration of 5.0×10^{7} cells/ml.

cold 0.09 M EDTA were added to each tube and the tubes were centrifuged. The sedimented cells were resuspended in 3.0 ml of 0.005 M EDTA-VB-saline or distilled water to obtain complete lysis. Subsequent treatment and the calculation were the same as in the preceding experiment.

As shown in Fig. 8, the initial velocities of S* formation increased with the C'3d concentration, and a linear relationship between the two was obtained within a certain range. However, the numbers of S* formed per unit time at a certain concentration of C'3d differed with different batches of cells. Therefore, this method cannot be used for measuring C'3d concentration unless a-cells with a large excess of a-sites are available. But C'3d concentrations can be compared with each other or with a standard series of dilution of a certain preparation of C'3d using a certain batch of a-cells in a single series of experiments.

7. *Depletion of C'3d by a-cells*

NISHIOKA and LINSCOTT (1963) reported that C'3d activity could not be removed from guinea pig serum by an antigen-antibody complex. They thought, therefore, that C'3d cannot participate in complement fixation and turns over from one site to another, acting like an enzyme. The experiments shown in Fig. 2, and especially those carried out at 0° and 30°C, showed that the values of $\vec{S}$* seemed to reach a certain value regardless of the concentration of C'3d added. These results and the Lineweaver-Burk's plot obtained suggest that the hypothesis that C'3d is an enzyme is reasonable. However, the experiments described above could be explained by the existence of excess C'3d in the reaction mixture, even when present at the lower concentration used and its relatively slow reactivity even if C'3d is actually depleted.

The measurement of the initial velocity of S* formation described in the previous paragraph provides a method for measurement of C'3d even supposing that the latter turns over

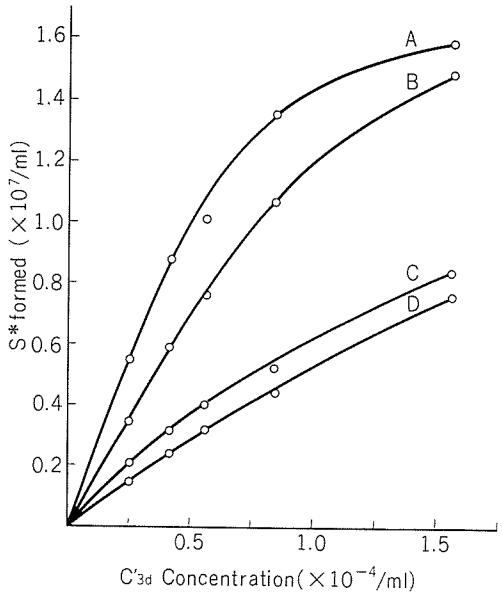

FIGURE 8 Relationship between the initial velocity and the C'3d concentration using a constant concentration of a-cells.

Za represents the maximal reactivity of the a-cells and is expressed by the value of S* formed with a large excess of C'3d.

Curve A: *Za*—1.609, Curve B: *Za*—1.487, Curve C: *Za*—0.759, Curve D: *Za*—0.721.

and its concentration is not depleted. Therefore, the possibility of C'3d depletion was re-examined.

Expt. 071966

C'3d (preparation #8) was diluted to 1/1,500 with 0.09 M EDTA and put into 6 tubes, which were then immersed in a water bath at 30°C. To each tube an equal volume of a-cell suspensions of varying concentrations (1.0×10^8 to 1.0×10^9 cells/ml) in 0.09 M EDTA or the same buffer without cells, at 30°C, was added and the contents were mixed well. The tubes were incubated with constant shaking for 60 min. After incubation the tubes were centrifuged in the cold. The supernatants (1st Sup.) showed no lysis and were reserved for measuring residual C'3d using the initial velocity method described in the previous paragraph with a single batch of a-cells. The sedimented cells were washed once with 10 ml of 0.09 M EDTA, and resuspended in 3.0 ml of 0.005 M EDTA-VB-saline. They were then incubated further at 30°C for 180 minutes. From the amount of hemoglobin released during this incubation period the number of S* formed in the first reaction step could be calculated.

In this experiment, with a constant amount of C'3d, the number of S* formed per cell was the same regardless of the concentration of the cells used, *i.e.*, total number of S* in each reaction mixture was proportional to the concentration of a-cells used, as shown in Fig. 9.

The C'3d activity left in the 1st Sup., on the other hand, decreased with the concentration of a-cells used. Only the relative amount of C'3d was measured so results could not be compared with the values of S* formed. However, there was a linear relationship between the concentration of a-cells added and both the amount of S* formed and the decrease in C'3d. This shows clearly that the amount of C'3d molecules in the reaction medium is depleted by a-sites resulting in the formation of S*.

8. *Non-transferability of C'3d from S**

The experiment described in the previous paragraph shows that C'3d can participated in complement fixation resulting in the formation of S*. However, it is possible that C'3d can be temporarily fixed on an a-site, and that, after converting the site to S*, C'3d can be transfered to another a-site at a slow turn-over

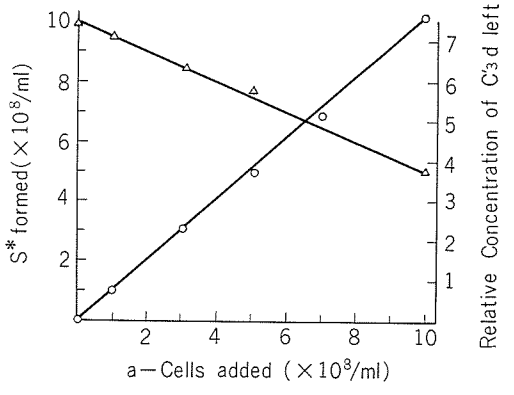

FIGURE 9 Depletion of C'3d by a-cells and formation of S*.

rate. To test this possibility the following experiment was carried out.

Expt. 100766

E* was formed by incubating a-cells with C'3d in 0.09 M EDTA for 5 min at 0°C. After the incubation the cells were diluted with about 500 volumes of cold 0.09 M EDTA and centrifuged rapidly in the cold for 5 min. The cells were washed similarly twice more. They were then resuspended in the same buffer at a concentration of 1.0×10^8 cells/ml, and delivered quickly into tubes containing varying amounts (0 to 1.0 ml) of a sample of a-cells in the same buffer. The total volume of each mixture was adjusted to 1.0 ml. These mixtures were incubated at 30°C for 90 min, with constant shaking. Then each tube received 10.0 ml of VB-saline and was incubated further at 37°C for 90 min. The tubes were centrifuged and the optical density of the supernatants was read at 413 mμ. As controls two similar series containing either E* or a-cells alone were included.

If active C'3d could be transfered from S* to an a-site, the amounts of lysed cells in the mixed cell system should exceed those in the system containing E* only. As shown in Fig. 10, however, the amounts of lysis were the same in both systems. Therefore, C'3d cannot be transfered from S*, even when this is formed by a short period of treatment of a-sites with C'3d in the cold.

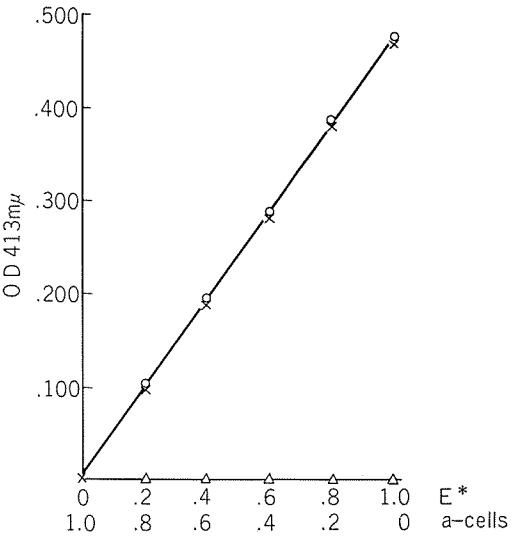

FIGURE 10 Possible transfer of C'3d from S* to a-sites.
×———×: the mixture of E* and a-cells,
○———○: E* only,
△———△: a-cells only.

9. *Titration of C'3d*

The experiments described above show that C'3d cannot be an enzyme-like substance turning over from one site to another but that its amount is depleted by a-sites resulting in the conversion of the latter to S*. Therefore, if a-cells containing sufficient excess a-sites are available, it should be possible to measure less than 4 effective molecules of C'3d per a-cell used after sufficient incubation of the mixture.

Expt. 100566

C'3 d (preparation #8) was diluted serially from 1/20,000 to 1/640,000 with 0.005 M EDTA-VB-saline. One ml of each dilution was mixed with an equal volume of a-cells at a concentration of 5.0×10^7 cells/ml and incubated at 30°C for 180 min. After incubation, 5.0 ml of cold VB-saline was added and tubes were centrifuged. The amounts of S* formed were calculated as $-\ln(1-y)$ from the amounts of oxyhemoglobin in the supernatants.

As shown in Fig. 11, a linear dose-response curve was obtained passing through the origin at lower concentrations of added C'3d. This gives evidence that the one-hit theory of MAYER (1961) can be extended to the last step of immune hemolysis. The concave curvature to the abscissa obtained at higher C'3d concentration suggests that the numbers of a-sites on the cells used were relatively small. As described earlier, a-sites decay at a relatively slow rate (the half life is about 800 min at 30°C), but S*'s do not decay. If a-cells with sufficient excess a-sites are available, the decay of a-sites can be ignored. At present, efforts are being made to prepare highly purified components of high-titer to prepare an intermediate of this type.

DISCUSSION

NISHIOKA and LINSCOTT (1963) and LINSCOTT

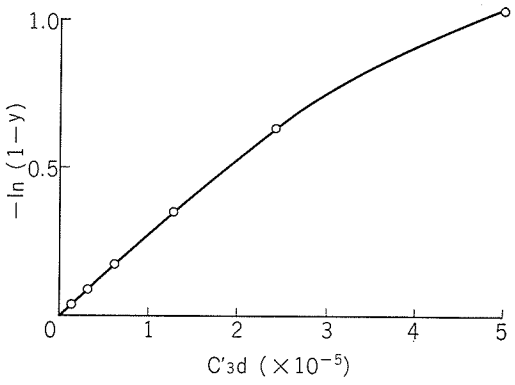

FIGURE 11 Titration of C'3d.

and NISHIOKA (1963) discovered a new component of complement in a partially purified in a partially purified C'2 preparation, prepared by Borsos' method (1961), during their study on the C'3 group of guinea pig complement. They termed it C'3d. Although they did not succeed in isolating it from guinea pig serum, they used a C'2 preparation prepared by Borsos' method or Cohn's fraction IV of bovine serum as a source of C'3d, and found that C'3d acted as the last complement component in immune hemolysis. Because of difficulties in preparing the intermediate, EAC'1, 4, 2, 3c, 3b, 3a, which became known as EAC'1, 4, 2, 3c, 3b, 3e, 3f, 3a (a-cells) after the discoveries of C'3e and C'3f (INOUE and NELSON, 1965 and 1966), they could not carry out experiment on C'3d depletion by a-cells. However, they treated guinea pig serum with the immune precipitate from egg albumin and rabbit anti-egg albumin antiserum, and detected no decrease in C'3d activity after treatment. They, therefore, thought that C'3d might well be enzymic in nature.

Our earlier experiments shown in Fig. 2, especially those carried out at 0°C and 30°C, suggested that the values of $\vec{S}^*$ reached an almost fixed value regardless of the concentration of C'3d used. These experiments supported the enzymic hypothesis of NISHIOKA and LINSCOTT. However, it seems that even the lower C'3d concentrations used in these experiments were rather too high, since C'3d depletion could not be achieved.

The experiment shown in Fig. 9 clearly shows that the amount of C'3d is reduced by a-sites, resulting in the conversion of the latter to S*. In this experiment the remaining C'3d activities in the supernatants were measured by the initial velocity method, which was based on the supposition of the enzymic nature of C'3d. Activities were found to decrease proportionally to the concentration of a-cells used. The total amount of S* formed in this experiment was also proportional to the cell concentration used. In other experiments using high dilutions of C'3d, (which are not shown in this paper), the decrease in residual C'3d was not linearly proportional to the concentration of a-cells used but gave a curve which was convex to the abscissa, and total amount of S* formed gave a curve which increased concavely to the abscissa. These differences may be mainly due to excess or insufficient C'3d used compared with the amount of a-sites available. The depletion of C'3d was also shown by measuring the C'3d left in the supernatant by the titration method described in the last paragraph in experiments which are also not shown in this paper. In this case the amount of C'3d decrease and that of total S* formed in each tube were closely comparable. These facts suggest that all the complement components from C'1 through C'3d can participate in the complement fixation reaction resulting in formation of the respective intermediate in the sequence.

The relationship of the activity of complement component and the ionic strength of the reaction medium has been thoroughly investigated. The formation of S* from a-sites was shown to be about the same at ionic strengths above 0.05 but decreased at lower ionic strengths. The lysis of E* was delayed at lower ionic strengths but this was shown not to be due to destruction of S* at lower ionic strength. In this respect sodium and potassium ion had similar effects, showing that the acceleration of lysis of E* at higher ionic

strength is not due to the qualitative effect of a certain ion on the equilibrium between intra- and extracellular cations.

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