# Ca2+とMg2+を必要とするコイ補体の2つの活性化段階および免疫溶血における中間生成物


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# Two Activation Steps of Carp Complement Requiring $Ca^{2+}$ and $Mg^{2+}$ and an Intermediate Product in Immune Hemolysis*1

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The action of carp complement on sensitized cells (EA) proceeds in two steps requiring $Ca^{2+}$ and $Mg^{2+}$, respectively. In order to decide which of these steps precedes in immune hemolysis, we carried out a kinetic experiment by use of a buffer and carp serum depleted of cations by passage through Amberlite IRC-50. In a reaction mixture composed of EA (sheep red blood cells sensitized with carp antibody), carp complement, optimal $Ca^{2+}$ and optimal $Mg^{2+}$, the hemolysis took place after a lag period of about 10 min, but when $Ca^{2+}$ was present at zero-time and $Mg^{2+}$ was added 20 min later, there was almost no lag. On the contrary, when $Mg^{2+}$ was present at zero-time and $Ca^{2+}$ was added 20 min later, there was a lag of about 20 min. These observations indicate that the step requiring $Ca^{2+}$ precedes that in which $Mg^{2+}$ functions. A hemolytic intermediate complex, which was provisionally designated EACx′, was prepared by treating EA with carp complement in the presence of $Ca^{2+}$, but with the absence of $Mg^{2+}$ at 20°C for 10 min. Furthermore, carp serum free from Cx′, which was provisionally designated Cy′, was also obtained by treating resin-passed carp serum with excessive EA in the presence of $Ca^{2+}$ but absence of $Mg^{2+}$ at 0°C for 3 h. It was confirmed that the cells in the state EACx′ were completely lysed when treated with a sufficient quantity of Cy′ and optimal $Mg^{2+}$, and that Cy′ did not react with EA even in the presence of optimal $Mg^{2+}$.

The term "complement" refers to a complex of enzymes in normal blood serum that, working together with antibodies or other factors, plays an important role in defending the animal against invading pathogens such as bacteria, viruses, fungi and other microorganisms.

The study on components of mammalian complement has been greatly advanced by MAYER and his co-workers,[1] who assumed that the action of guinea pig complement on sensitized cells (EA) takes place in two successive steps. The first of these requires $Ca^{2+}$ and involves the fixation of certain components (Cx) of complement with formation of a complex EACx, and in the second step, which requires $Mg^{2+}$, the complex EACx reacts with the other components (Cy) of complement. On the basis of this assumption, LEVINE and MAYER[2] isolated EACx and Cy by treating EA with complement in the presence of $Ca^{2+}$, but absence of $Mg^{2+}$, and subsequently identified the complement components involved in Cx and Cy.

It has been known since 1945[3] that teleosts possess a complement system which is similar to that of mammals. ROSS and JENSEN[4,5] reported their findings of the first component (C1) of nurse shark complement, and NONAKA *et al.*[6,7] reported that they purified the fifth component (C5) of rainbow trout complement. However, there is little information about the kinetics of immune hemolysis and it is not yet clarified how many components take part in the action of fish complement on sensitized cells (EA).

We started a series of studies to give the entire picture of carp complement system. In the previous paper,[8] we confirmed that both $Ca^{2+}$ and $Mg^{2+}$ were essential for the activation of carp complement, and that their optimum concentrations in hemolytic reaction were nearly equal to those described for mammals.

The present investigation was undertaken to decide which of the two reaction steps requiring $Ca^{2+}$ and $Mg^{2+}$ precedes in immune hemolysis and to isolate a hemolytic intermediate complex corresponding to mammalian EACx.

## Materials and Methods

*Reagents*

Amberlite IRC-50 was purchased from Organo Co., Tokyo. MS-222 (tricain methanesulfonate)

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was purchased from Sankyo Co., Tokyo and barbital sodium from Wako Pure Chemical Industries Co., Osaka. Sheep blood in Alsever's solution was obtained from Japan Bio-Supply Center, Tokyo.

The buffers used in this experiment and their abbreviations are: GVB ($Ca^{2+}$), Veronal-buffered saline, pH 7.4, containing 0.1% gelatin and 0.15 mM $CaCl_2$; GGVB ($Ca^{2+}$), Veronal-buffered saline, pH 7.4, containing 2.5% glucose, 0.1% gelatin and 0.15 mM $CaCl_2$; EDTA·GVB, Veronal-buffered saline, pH 7.4, containing 0.1% gelatin and 0.01 M EDTA; GVB (f), Veronal-buffered saline free from divalent cations, pH 7.4, containing 0.1% gelatin. GVB (f) was made by passing 200 m*l* of Veronal-buffered saline containing 0.1% gelatin through Amberlite IRC-50 (5 m*l*) and readjusting its pH to 7.4.

*Preparation of Carp Normal Serum*

Carps, weighing 650 to 880 g, were purchased from a fish farm and maintained in aquaria (25–28°C) for more than a month. For bleeding, fish was anesthetized in MS-222 solution, and ca. 8 m*l* of blood was collected from the dorsal aorta of fish without anticoagulant. The blood was allowed to clot at 30°C for 30 min and cooled at 0°C for 2 h. After centrifugation at 3000 rpm for 10 min, the supernatant serum was stored at −35°C until use.

*Preparation of Anti-SRBC Carp Serum*

A carp was given an intraperitoneal injection of 0.2 m*l* of sheep red blood cell (SRBC) stroma suspension (1 mgN/m*l*) which was prepared according to the method described in the previous paper.[8] Injection was repeated 5 times at 5 day intervals over a period of 20 days and the carp was bled on the 5th day after the last injection. After centrifugation, the supernatant antiserum was inactivated at 50°C for 20 min[8] and stored at −35°C.

*Preparation of Carp Serum Deficient in Divalent Cations*

Pooled carp normal serum was passed through a cation exchange resin to remove divalent cations. A micro-column of Amberlite IRC–50 (1×4 cm) was prepared in a 3 m*l* syringe fitted with a 25 G needle and washed with 0.15 M NaCl. Aliquot (0.5 m*l*) of carp serum was applied to the column and eluted with 2.75 m*l* of GVB (f). The first eluate (0.25 m*l*) was discarded and the next eluate (2.5 m*l*) was used as the carp serum deficient in divalent cations [serum (f)].

*Preparation of SRBC Sensitized with Carp Antibody (EA)*

Sheep red blood cells (SRBC) in Alsever's solution were washed twice with GVB (f) and suspended in the same buffer to a concentration of $1\times10^9$/m*l*. Anti-SRBC carp serum was optimally diluted (1:100 to 1:200) with EDTA·GVB. Equal volumes of the SRBC suspension and of the diluted antiserum were mixed up and incubated at 30°C for 20 min with occasional shakes. The mixture was cooled and centrifuged at 2000 rpm for 5 min and the precipitate (EA) was washed three times with GVB (f) and resuspended in the same buffer to a concentration of $5\times10^8$/m*l*.

*Time Course of Hemolytic Reaction in the Presence of both $Ca^{2+}$ and $Mg^{2+}$*

Serum (f) was previously diluted (1:2 to 1:3) with GVB (f) to give 70 to 80% lysis under optimal conditions, and hemolytic reaction was carried out in a total volume of 1.5 m*l*. Ten identical reaction mixtures composed of 0.2 m*l* of EA suspension ($5\times10^8$/m*l*), 0.5 m*l* of diluted serum (f), 15 $\mu l$ of $CaCl_2$ (15 mM), 15 $\mu l$ of $MgCl_2$ (50 mM) and 0.77 m*l* of GVB (f) were incubated at 30°C in small test tubes. At timed intervals, a test tube was taken out and centrifuged at 3000 rpm for 5 min. The hemolysis rate was calculated from optical density at 541 nm ($OD_{541}$) of the supernatant. A control, containing EA and GVB (f), was included to check the stability of the cells. The complete lysis of $1\times10^8$ EA gave an $OD_{541}$ reading of 0.340 when measured with a spectrophotometer (Shimazu, UV-240) equipped with 0.5 cm light-path cuvettes.

*Effect of Delayed Addition of $Mg^{2+}$ on the Time Course of Hemolysis*

Eight identical reaction mixtures containing 0.2 m*l* of EA suspension ($5\times10^8$/m*l*), 0.5 m*l* of diluted serum (f), 15 $\mu l$ of $CaCl_2$ (15 mM) and 0.77 m*l* of GVB (f) were incubated at 30°C in small test tubes. After 20 min, 15 $\mu l$ of $MgCl_2$ (50 mM) was added to each tube and incubation was continued. At timed intervals, a test tube was taken out and centrifuged at 3000 rpm for 5 min. The degree of hemolysis was calculated from $OD_{541}$ of the supernatant.

*Effect of Delayed Addition of $Ca^{2+}$ on the Time Course of Hemolysis*

Ten reaction mixtures containing 0.2 m*l* of EA suspension, 0.5 m*l* of diluted serum (f),

15 $\mu l$ of $MgCl_2$ (50 mM) and 0.77 m$l$ of GVB (f) were incubated at 30°C for 20 min, followed by addition of 15 $\mu l$ of $CaCl_2$ (15 mM) to each tube. The incubation was continued and the hemolysis rate was measured at timed intervals as described above.

*Preparation of EACx′*

A hemolytic intermediate complex EACx′, which corresponds to mammalian EACx, was prepared by treating carp antibody-sensitized SRBC (EA) with serum (f) in the presence of $Ca^{2+}$, but absence of $Mg^{2+}$.

Serum (f) was previously diluted with GVB (f) to give a complement concentration of 2 $CH_{50}$ units/m$l$. Four m$l$ of this diluted serum (f) was mixed with 2 m$l$ of EA suspension ($5\times10^8$/m$l$), followed by addition of 60 $\mu l$ of $CaCl_2$ (15 mM), and the mixture was incubated at 20°C for 10 min. The cells (EACx′) were collected by centrifugation at 2000 rpm for 3 min, washed twice with GVB ($Ca^{2+}$) and resuspended in 2 m$l$ of GGVB ($Ca^{2+}$).

*Preparation of Cy′*

Six m$l$ of EA suspension ($5\times10^8$/m$l$) was centrifuged at 2000 rpm for 5 min. To the sedimented cells were added 3 m$l$ of the serum (f), which had been diluted with GVB (f) to give a complement concentration of 2 $CH_{50}$ units/m$l$, and 30 $\mu l$ of $CaCl_2$ (15 mM). The mixture was kept at 0°C for 3 h with occasional shakes, centrifugaed at 2000 rpm for 5 min, and the supernatant (Cy′) was stored at 0°C until use.

The time required for the complete removal of Cx′ from carp serum was determined as follows: Every 30 to 60 min during the incubation, 0.3 m$l$ aliquot was removed from the reaction mixture and centrifuged at 2000 rpm for 5 min, then 0.2 m$l$ of the supernatant was pipetted into a mixture composed of 0.2 m$l$ of EA suspension ($5\times10^8$/m$l$), 15 $\mu l$ of $CaCl_2$ (15 mM), 15 $\mu l$ of $MgCl_2$ (50 mM) and 1.07 m$l$ of GVB (f). The mixture was then incubated at 30°C for 60 min and the hemolysis rate was measured as usual.

*Reaction between EACx′ and Cy′*

In order to confirm the formation of EACx′ and Cy′, five reaction mixtures were set up according to the following table.

Each reaction mixture was incubated at 30°C for 60 min in a small test tube. The degree of hemolysis was determined by spectrophotometric analysis for oxyhemoglobin ($OD_{541}$).

| test tube | A | B | C | D | E |
|---|---|---|---|---|---|
| EACx′ suspen., m$l$ | 0.20 | 0.20 | 0.20 | — | — |
| EA suspen., m$l$ | — | — | — | 0.20 | 0.20 |
| Cy′, m$l$ | 0.20 | 0.20 | — | 0.20 | — |
| 75 mM $MgCl_2$, m$l$ | 0.01 | — | 0.01 | 0.01 | 0.01 |
| GVB (f), m$l$ | 1.09 | 1.10 | 1.29 | 1.09 | 1.29 |
| Total, m$l$ | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 |

* EACx′ and EA were suspended in GVB(f) to a concentration of $5\times10^8$/m$l$.

## Results

*Effect of Resin Treatment on Complement Activity*

As described in Materials and Methods, carp serum was diluted 1/5 through Amberlite IRC-50 treatment. The recovery of the hemolytic complement activity from the resin was approximately 70%, and the values for the constant 1/n in the Von Krogh equation (which describes the shape of the dose-response curve for complement hemolysis) did not change by a passage through the cation exchange resin, indicating that the properties of the complement have not been altered. The respective concentrations of $Ca^{2+}$ and $Mg^{2+}$ in serum (f) were less than $1\times10^{-6}$ M, when measured by atomic absorption spectrophotometer (Nippon Jarrel-Ash Co., AA-500).

*Time Course of Hemolysis in the Presence of both $Ca^{2+}$ and $Mg^{2+}$*

Curve-A in Fig. 1 shows the time course of hemolytic reaction in the presence of $Ca^{2+}$ and $Mg^{2+}$. The hemolysis did not commence immediately upon addition of complement to EA, but took place after a lag period of about 10 min. This lag would be attributable to the time required for the first reaction step. After the lag period, hemolysis rate gradually increased with time displaying a sigmoidal response curve and reached a plateu in about 50 min.

*Effect of Delayed Addition of $Ca^{2+}$ (or $Mg^{2+}$) on the Time Course of Hemolysis*

In order to decide which of the two reaction steps requiring $Ca^{2+}$ and $Mg^{2+}$ precedes in immune hemolysis, we carried out a kinetic experiment considering that if EA are allowed to react with complement in the presence of $Ca^{2+}$, but absence of $Mg^{2+}$, initiation of lysis on addition of $Mg^{2+}$ will take place without lag, provided the sequence of reaction steps is $Ca^{2+}$ followed by $Mg^{2+}$. Conversely, if the reaction step sequence is $Mg^{2+}$

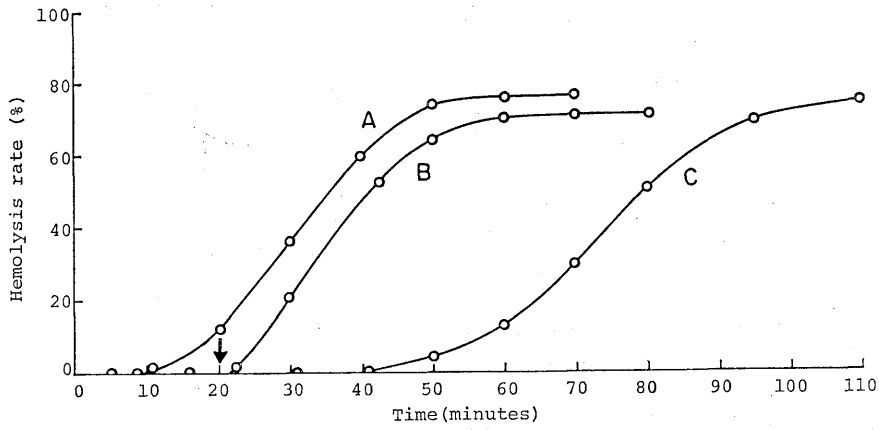


**Fig. 1.** Effect of delayed addition of $Ca^{2+}$ or $Mg^{2+}$ on the time course of hemolysis of EA by carp complement.

When both $Ca^{2+}$ and $Mg^{2+}$ were present at zero-time, there was a lag period of about 10 min (Curve-A). When $Ca^{2+}$ was present at zero-time and $Mg^{2+}$ was added 20 min later, hemolysis began almost immediately (Curve-B). When $Mg^{2+}$ was present at zero-time and $Ca^{2+}$ was added 20 min later, the lag period was about 20 min (Curve-C). An arrow in the figure means the time of addition of $Ca^{2+}$ or $Mg^{2+}$.

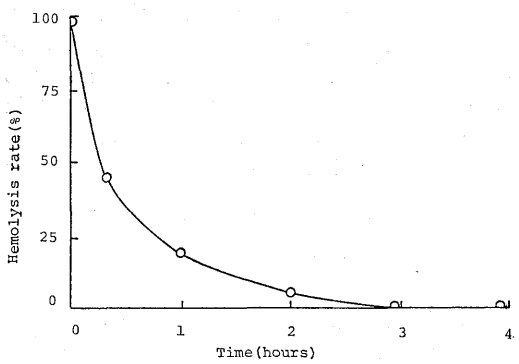


**Fig. 2.** Determination of the incubation time required for the preparation of Cy′.

Resin-passed serum was incubated at 0°C with excessive EA in the presence of $Ca^{2+}$, and the residual hemolytic activity was measured.

followed by $Ca^{2+}$, a lytic reaction conducted in the presence of $Mg^{2+}$ but absence of $Ca^{2+}$, will take place promptly upon addition of $Ca^{2+}$.

Curve-B in Fig. 1 shows the time course of hemolytic reaction when $Ca^{2+}$ was present at zero-time and $Mg^{2+}$ was added 20 min after introduction of complement. As seen in the figure, there was almost no lag (less than 3 min). On the contrary, when $Mg^{2+}$ was present at zero-time and $Ca^{2+}$ was added 20 min after introduction of complement, there was a lag of about 20 min following addition of $Ca^{2+}$ (Curve-C in Fig. 1). These observations indicate that the sensitized cells first react with certain components of complement in the reaction step involving $Ca^{2+}$, and that the $Mg^{2+}$ step cannot get under way until the preceding $Ca^{2+}$ step has taken place.

*Preparation of EACx′ and Cy′*

A hemolytic intermediate, EACx′, was produced by treating EA with serum (f) at 20°C in the presence of $Ca^{2+}$, but absence of $Mg^{2+}$. After 10 minutes' reaction the mixture was centrifuged, and after removal of the fluid phase, the cells, which were in the state EACx′, were washed and suspended in GGVB ($Ca^{2+}$). EACx′ thus obtained was stable on washing, and retained its activity for at least two days when stored at 0°C, provided $Ca^{2+}$ was present. Treatment of carp serum (f) with excessive EA in the presence of $Ca^{2+}$ but absence of $Mg^{2+}$ removed all detectable Cx′, and left most of the Cy′ in the fluid phase. As seen in Fig. 2, the time required for the complete removal of Cx′ from Cy′ was ca. 3 h at 0°C.

*Reaction between EACx′ and Cy′*

In order to check the properties of EACx′ and Cy′, five reaction mixtures (Tube A, B, C, D, E) were set up as described in the section of Materials and Methods. Tube A represents the reaction between EACx′ and Cy′ in the presence of optimal $Mg^{2+}$. Tube B represents the reaction between EACx′ and Cy′ in a $Mg^{2+}$ deficient system. Tube C serves as a control for the stability of EACx′. Tube D represents the reaction between EA and Cy′ in the presence of optimal $Mg^{2+}$ and Tube E

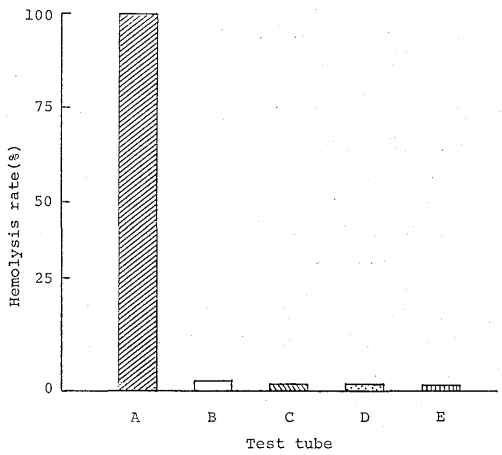


**Fig. 3.** Properties of EACx′ and Cy′.
Tube A represents the reaction between EACx′ and Cy′ in the presence of $Mg^{2+}$; Tube B, the reaction between EACx′ and Cy′ in a $Mg^{2+}$ deficient system; Tube C, a control for the stability of EACx′; Tube D, the reaction between EA and Cy′ in the presence of $Mg^{2+}$; Tube E, a control for the stability of EA.

is the corresponding control.

As shown in Fig. 3, Tube A showed 100% lysis, while Tube B showed only about 3% lysis, indicating that the reaction between EACx′ and Cy′ proceeds only in the presence of $Mg^{2+}$. Control Tube C showed approximately 2% hemolysis, indicating that the cells in the state EACx′ do not undergo spontaneous lysis to an extent significantly greater than that of cells in the state EA (Tube E). Similarly, control Tube D showed only about 2% hemolysis after 1 h, as compared with approximately 1.5% lysis in control Tube E, establishing that Cy′ did not react with EA.

## Discussion

The studies on the complement system of fish have been hampered by technical difficulties arising from interspecies incompatibility. It is said that complement proteins of fish species can rarely be assayed by well-defined antibody and complement reagents of mammalian species. The interspecies incompartibility was also confirmed in our preliminary study, *i.e.*, the hemolytic activity of carp complement could not be measured with rabbit antibody-sensitized SRBC, and on the contrary carp antibody-sensisized SRBC did neither combine nor activate guinea pig (or human) complement. Therefore, in this paper, all the hemolytic reactions were performed by use of carp antibody-sensitized SRBC.

The present study clearly demonstrated that $Ca^{2+}$ step precedes $Mg^{2+}$ step in the hemolytic action of carp complement. The kinetic experiment presented in Fig. 1 provides a clear-cut answer to the question of sequence of reaction steps. However, it poses a question which cannot be answered at present. Why does Curve-C display a longer lag period than Curve-A? This sort of prolongation of lag period was also reported by LEVINE *et al.*[1], who observed that preliminary incubation of sensitized red blood cells and guinea pig complement with $Mg^{2+}$, prior to the addition of $Ca^{2+}$, led to a prolongation of the lag period. The reason for this has not been clarified yet.

Our finding of the two successive reaction steps in immune hemolysis has a great significance for the study of components of carp complement. Now it became possible to separate carp complement into two groups, one requiring $Ca^{2+}$ and the other requiring $Mg^{2+}$. With this view, we have tried to prepare a hemolytic intermediate complex in the presence of $Ca^{2+}$, but absence of $Mg^{2+}$.

As expected, an intermediate complex EACx′, which corresponds to mammalian EACx, was obtained by incubating EA with serum (f) in the presence of optimal $Ca^{2+}$ at 20°C for 10 min. EACx′ was also obtained at 30°C, but a more reactive EACx′ was obtained at 20°C. Furthermore, Cy′ (carp serum free from Cx′) was obtained by incubation of serum (f) with excessive EA and optimal $Ca^{2+}$ at 0°C for 3 h. LEVINE and MAYER[2] used albumin-anti-albumin complex to remove Cx from Cy, but we succeeded in obtaining Cy′ by use of EA as a substitute. Cy′ could be prepared at higher temperatures. However, the preparation made at 30°C appeared to be much more labile than that prepared at 0°C. It has also been shown in the present work that EACx′ are completely lysed when treated with a sufficient quantity of Cy′, indicating that all the cells are in the state EACx′. Furthermore, it has been found that EACx′ does not undergo spontaneous lysis upon incubation at 30°C for 60 min. On the contrary, Cy′ did not react with EA even in the presence of optimal $Mg^{2+}$, indicating the complete removal of Cx′ from Cy′. When stored in GGVB ($Ca^{2+}$), EACx′ was stable at least 2 days, but it rapidly lost its activity in GVB (f), suggesting that $Ca^{2+}$

is indispensable for the stabilization of the complex. Cy′ maintained its activity for 3 days at 0°C, while the original serum lost 37% of its activity in a day[8].

Finally, it is of quite interest to learn how many components of carp complement are involved in EACx′ and Cy′, respectively, since mammalian EACx is known to involve two components (C1, C4), whereas Cy is composed of seven components (C2, C3, C5, C6, C7, C8, C9). This problem will be dealt with in detail in the following paper.

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