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# An Improved Method for the Preparation of EAC14, an Intermediate in Immune Hemolysis by Carp Complement

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We have succeeded in preparing EAC14 (sheep erythrocytes sensitized with carp antibody and complement components, Cl and C4) by incubating EA with carp serum in the presence of 5 mM TTHA (triethylenetetramine-*N,N,N',N'',N''',N'''*-hexaacetic acid), a selective chelator of $Mg^{2+}$, and 5 mM $CaCl_2$ at pH 7.4. The optimal conditions for preparing EAC14 were as follows: ionic strength, 0.085; reaction temperature, 25°C; incubation period, 10 min. The EAC14 prepared by this method was stable for at least 4 days when stored at 4°C in $GGVB^{2+}$ (veronal-buffered saline, pH 7.4, containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose and 0.02% $NaN_3$).

## INTRODUCTION

In mammals, EAC14 (sheep erythrocytes sensitized with antibody and complement components, Cl and C4) is used not only for the titration of C2, but also as a starting material for the preparation of other hemolytic intermediate complexes.

In previous papers (Yano et *al.*, 1985, 1986), we demonstrated that immune hemolysis of EA (sheep erythrocytes sensitized with carp antibody) by carp complement proceeds in two successive steps. The first step requires $Ca^{2+}$ and involves the fixation of carp Cl and C4, and in the second step which necessitates $Mg^{2+}$, the complex EAC14 reacts with the other complement components. We obtained carp EAC14 by incubating EA with $Mg^{2+}$-depleted carp serum which was prepared by allowing carp serum to pass through an Amberlite IRC-50 (Na-form) column to remove divalent cations and by adding $Ca^{2+}$. However, the chromatographic procedure was rather complicated and took a lot of time.

In this work, we attempted to prepare carp EAC14 by incubating EA with carp serum in the presence of TTHA (triethylenetetramine-*N,N,N',N'',N''',N'''*-hexaacetic acid), a selective chelator of $Mg^{2+}$.

## MATERIALS AND METHODS

### Buffers

The buffers used in this experiment and their abbreviations were as follows: $GGVB^{2+}$, veronal-buffered saline (pH 7.4) containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose and 0.02% $NaN_3$; EDTA•GVB, veronallbuffered saline

(pH 7.4) containing 0.1% gelatin and 10 mM EDTA; Ca•TTHA•GGVB, veronal-buffered saline (pH 7.4) containing 0.1% gelatin, 2.5% glucose, 5 mM $CaCl_2$ and 5 mM TTHA (Tokyo Kasei Kogyo Co., Tokyo).

**Collection of carp serum**

Blood was collected from carp *Cyprinus carpio* by peduncle amputation, allowed to clot at room temperature for 30 min and cooled at 0°C for 1 h. The serum was then separated by centrifugation at 3,000 rpm for 10 min and stored in small aliquots at -80°C until use.

**Preparation of ammonia-treated carp serum (Am)**

Carp serum was treated with ammonia in the same manner as described previously (Yano et al., 1986) and diluted 1/30 with ice-cold $GGVB^{2+}$. This inactivated serum (Am) was used within one hour.

**Preparation of EA**

Sheep red blood cells (SRBC) in Alsever's solution (Japan Bio-Supply Center, Tokyo) were washed with EDTA•GVB, standardized spectrophotometrically to a concentration of $1 \times 10^9$ cells/ml, and sensitized with an equal volume of an appropriate dilution of carp antiserum at 25°C for 30 min. The sensitized SRBC (EA) formed were washed with $GGVB^{2+}$ and brought up to a final concentration of $5 \times 10^8$ cells/ml.

**Determination of the optimal conditions for the preparation of EAC14**

**1)** Concentration of calcium

Calcium concentration of Ca•TTHA•GGVB was adjusted to 1, 3, 5, 7 and 10 mM by changing the concentration of $CaCl_2$. EA was washed twice and suspended in above buffers to $5 \times 10^8$ cells/ml. Carp serum was diluted with above buffers to 2 CH50 units/ml and preincubated at 20°C for 10 min. Four ml of each diluted serum was incubated with 2 ml of EA suspension ($5 \times 10^8$ cells/ml) in the corresponding buffer at 20°C for 15 min. After centrifugation at 1,800 rpm for 5 min, the precipitate (EAC14) was washed twice with respective Ca•TTHA•GGVB and twice with $GGVB^{2+}$, and then suspended in $GGVB^{2+}$ to $3 \times 10^8$ cells/ml.

To 0.1 ml of each EAC14 suspension ($3 \times 10^8$ cells/ml) was added 0.5 ml of Am (carp serum deficient in C4) and 0.9 ml of $GGVB^{2+}$ and the mixture was incubated at 25°C for 30 min. After centrifugation, the degree of hemolysis was calculated by reading the absorbance at 414 nm of the supernatant.

2) Ionic strength

Ionic strength of Ca•TTHA•GGVB was adjusted to 0.029, 0.048, 0.085, 0.12 and 0.16 by changing the concentration of NaCl, the osmotic pressure being kept at isotonic by regulating the concentration of glucose. Carp serum was diluted with above buffers to 2 CH50 units/ml and 4 ml of each diluted serum was incubated with 2 ml of EA suspension ($5 \times 10^8$ cells/ml) in the corresponding buffer at 20°C for 15 min. EAC14 thus obtained was incubated with Am and the degree of hemolysis was measured in the same manner as above.

3) Reaction temperature

Carp serum was diluted with Ca•TTHA•GGVB to 2 CH50 units/ml. Four ml of

the diluted serum was incubated with 2 ml of EA suspension (5 x $10^8$ cells/ml) in Ca•TTHA•GGVB at 0, 10, 20, 25 and 30°C for 15 min. EAC14 thus obtained was combined with Am and the degree of hemolysis was measured in the same manner as above.

4) Incubation period

Carp serum was diluted with Ca•TTHA•GGVB to 2 CH50 units/ml. Four ml of the diluted serum was incubated with 2 ml of EA suspension (5 x $10^8$ cells/ml) in Ca•TTHA•GGVB at 25°C for 3, 5, 10, 15 and 30 min. EAC14 thus obtained was combined with Am and the degree of hemolysis was measured in the same manner as above.

## RESULTS

### Optimal conditions for the preparation of EAC14

In order to determine the optimal conditions for the preparation of EAC14, EA was incubated with carp serum at different calcium concentrations, ionic strengths and incubation temperatures for different incubation periods.

1) Concentration of calcium

When EA was incubated at different concentrarions of $CaCl_2$ (1, 3, 5, 7 and 10 mM) at 20°C for 15 min, the formation of EAC14 was maximum at 5 mM $CaCl_2$ (Fig. 1).

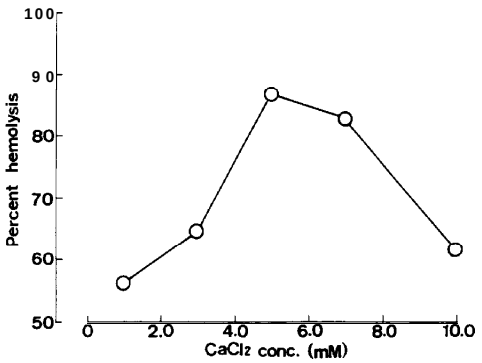



**Fig. 1.** Effect of concentration of calcium on the formation of EAC14. The hemolytic reaction was performed at 25°C for 30 min.

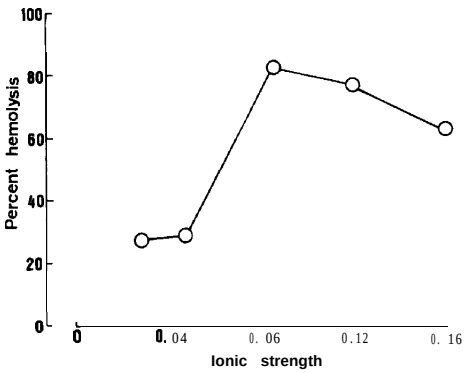



**Fig. 2.** Effect of ionic strength on the formation of EAC14. The hemolytic reaction was performed at 25°C for 30 min.

2) Ionic strength

When EA was incubated at different ionic strengths (0.029, 0.048, 0.085, 0.12 and 0.16) at 20°C for 15 min, the formation of EAC14 was maximum at an ionic strength 0.085, but was greatly reduced at ionic strengths 0.048 and below (Fig. 2).

3) Reaction temperature

When EA was incubated at different temperatures (0, 10, 20, 25 and 30°C) for 15 min, the formation of EAC14 was maximum at 25°C (Fig. 3). It was also found that a considerable amount of EAC14 was obtained even at 0°C.

4) Incubation period

When EA was incubated at 25°C for 3, 5, 10, 15 and 30 min, percent hemolysis increased with the increasing incubation period and reached a plateau in about 10 min (Fig. 4).

In conclusion, the optimal conditions were determined as follows: concentration of calcium, 5 mM; ionic strength, 0.085; reaction temperature, 25°C; incubation period, 10 min. Fig. 5 represents the scheme for the preparation of EAC14.

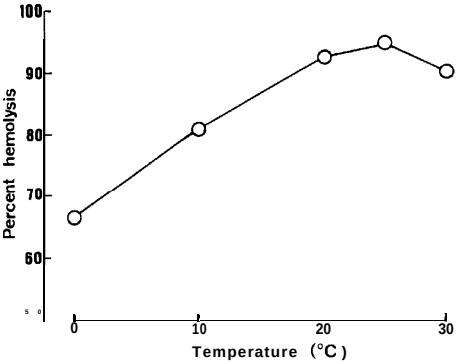



Fig. 3. Effect of reaction temperature on the formation of EAC14. The hemolytic reaction was performed at 25°C for 30 min.

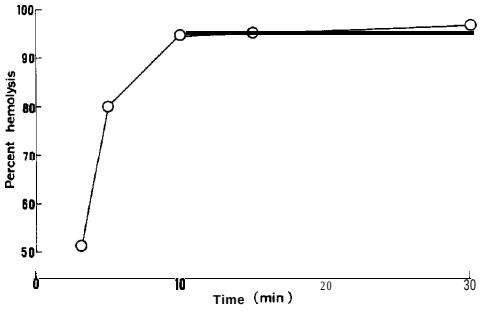
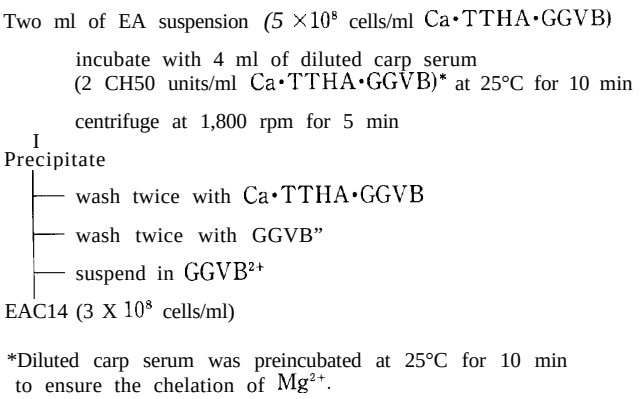



Fig. 4. Effect of incubation period on the formation of EAC14. The hemolytic reaction was performed at 25°C for 30 min.




*Diluted carp serum was preincubated at 25°C for 10 min to ensure the chelation of $Mg^{2+}$.

**Fig. 5.** Scheme for the preparation of carp EAC14.

**Stability of EAC14**

EAC14 was stored at 4°C for 7 days in $GGVB^{2+}$. On days 1, 2, 4 and 7, the EAC14 was incubated with Am and percent hemolysis was measured. The results showed that EAC14 was stable for at least 4 days, but its activity decreased to 91% on the 7th day (data not shown).

## DISCUSSION

TTHA is a chelating agent which has a stability constant (log k) of 13.93 for $Mg^{2+}$ and 9.89 for $Ca^{2+}$. Utilizing this difference it is possible to obtain $Mg^{2+}$-depleted serum in the presence of $Ca^{2+}$. Nagaki et al., (1974) prepared human EAC14 by incubating EA with human serum in the presence of 5 mM TTHA and 5 mM $CaCl_2$ at pH 5.1-5.2, at ionic strength 0.085, at 30°C for 5 min.

We have shown in this work that this method is applicable to carp complement with some modifications. In contrast to human complement which formed EAC14 at pH 5.1-5.2, carp complement did not react with EA at pH in the acidic region; carp EAC14 was obtained only in the neutral region. This is probably due to the unstability of carp complement under acidic condition. In carp, the optimal incubation temperature was 25°C and a considerable amount of EAC14 formed even at 0°C. This is in agreement with our observation that carp complement works at 0°C (unpublished data).

The EAC14 obtained in this experiment did not hemolyze when combined with heattinactivated carp serum (serum deficient in Cl and C2) and hydrazine-treated carp serum (serum deficient in C4 and C3) (Yano et al., 1986). This indicates that the cells did not include C2 and late-acting components.

The present method has some advantages when compared with our old method in which we used Amberlite IRC-50 to remove divalent cations. It is simple and speedy and does not require the chromatographic procedure. Indeed, EAC14 can be obtained within 1.5 h.

This simple method will promote the research on carp complement, since EAC14 can be utilized as target cells in the assay of C2 and C3 components, and as a starting

material for the preparation of other hemolytic intermediates.

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