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Optimum Conditions for the Assay of Hemolytic Complement Titer of Carp and Seasonal Variation of the Titers

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The optimum conditions for the assay of the hemolytic complement titer (CH_{50}, unit/ml) of carp, *Cyprinus carpio*, was investigated by use of sheep red blood ceils (SRBC) sensitized with carp antiserum. The antiserum was obtained from carp immunized with SRBC stromata for 25 days. The heat inactivation of complement which coexists within the antiserum was performed at 50°C for 20 min. The optimum conditions for hemolytic complement reaction were confirmed as follows: reaction temperature, 25°C; pH, 7.2 to 8.5; concentrations of Ca^{2+} and Mg^{2+}, 0.1 to 0.5 mM and 0.5 to 1.0 mM, respectively. These results show that the optimum conditions for assaying complement activity of carp are quite similar to those of mammals, and that Mayer's method (37°C, pH 7.4, 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}) is applicable to carp by lowering the reaction temperature from 37°C to 30°C or below. The complement titers of carps which were obtained from fish farms located in the south of Fukuoka Prefecture, were estimated from September in 1983 till May in 1984. The complement titers gradually decreased as the temperature fell in winter. In spring, the titers began to increase, and again reached high values in May.

INTRODUCTION

The complement system of vertebrate is composed of a series of enzymes which are normally present in fresh serum and play an important role in defending the animal against various infections, i.e., the enzymes react with invading micro-organisms such as bacterium, protozoa and virus and exclude them from body in combination with antibody (classical pathway) or independently (alternative pathway).

The hemolytic complement activity of mammals is usually assayed by Mayer's method (1961). In Mayer's method, one unit of CH_{50} refers to the amount of complement which needs to hemolyse 50 % of 5×10^8 sheep red blood cells in 7.5 ml of isotonic buffer (pH 7.4) containing optimum concentrations of Ca^{2+} (0.15 mM) and Mg^{2+} (0.5 mM), at 37°C for 60 min.

Mammalian antibody does not activate fish complement (Legler and Evans, 1967 a; Ross and Jensen, 1973; Sakai, 1981). Therefore, in order to assay the hemolytic complement activity of fish, one has to prepare red blood cells sensitized with antibody derived from fish. Legler and Evans (1976 a, b) uti-

lized sheep red blood cells sensitized with natural antibody of fish or turtle antibody for the determination of hemolytic activities of various fish sera. Day et al. (1970) employed rabbit red blood cells sensitized with carp natural antibody. Sakai (1981) used red blood cells of goldfish, rainbow trout, rabbit and sheep sensitized with immune antibody produced by rainbow trout or goldfish. Thus the method for titrating hemolytic complement activity of fish differs among investigators, i. e., they used different kinds of red blood cells and antibodies, and carried out the hemolytic reaction at different temperatures for different reaction times.

The authors investigated the optimum conditions for assaying hemolytic complement titer of carp by use of sheep red blood cells sensitized with the immune antibody produced by carp, and subsequently examined the seasonal change of complement titers of carps which were bred in outdoor crawls of fish farms.

MATERIALS AND METHODS

1. Reagents

The source of reagents used in the present work are as follows: EDTA (ethylenediamine tetraacetic acid, disodium salt) and barbital sodium, Wako Pure Chemical Industries Co., Osaka ; merthiolate (sodium ethylmercurithiosalicylate), Katayama Chemical Industries Co., Osaka; MS-222 (tricain methanesulfonate), Sankyo Co., Tokyo; Amberlite IRC-50, Organo Co., Tokyo; sheep blood in Alsever's solution, Japan Bio-Supply Center, Tokyo.

The buffers used in the experiment and their abbreviations are: GVB^{2+}, Veronal-buffered saline, pH 7.4, containing 0.1% gelatin, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$; EDTA•GVB, Veronal-buffered saline containing 0.01 M EDTA and 0.1% gelatin; GGVB, Veronal-buffered saline containing 2.5 % glucose, 0.1% gelatin; GVB(f), Veronal-buffered saline free from divalent cations, pH 7.4, containing 0.1% gelatin. GVB(f) was made by passing 200 ml of Veronal-buffered saline containing 0.1% gelatin through Amberlite IRC-50 (5 ml) and readjusting its pH to 7.4. Except where noted, GVB^{2+} was employed in this experiment.

2. Fish

Carp, *Cyprinus Carpio*, weighing 500–700 g were purchased from a fish farm near Fukuoka City. Prior to use in the experiment the fishes were maintained in aquaria for more than a month at a water temperature of 24±1℃ and on a 14 L/10 D photoregime.

3. Immunogen

In order to immunize carp against sheep red blood cells (SRBC), SRBC stroma suspension was prepared as immunogen according to Nishioka (1966).

One hundred ml of sheep blood in Alsever's solution (1 : 1) was centrifuged at 3000rpm for 10 min, the precipitate was washed twice with 200ml of

physiological saline (0.85 % NaCl) by centrifuging at 3000 rpm for 5 min and then hemolysed in 1 liter of distilled water containing 0.4 ml of acetic acid. The suspension was allowed to stand overnight at 5°C, the supernatant was carefully decanted and residual stromata were washed 6 times with 0.01 M acetate buffer (pH 5.0) and once with saline, centrifuged at 3000 rpm for 20 min and finally resuspended in 30 ml of saline using a glass-teflon homogenizer.

The content of N in the stroma suspension was assayed by micro-Kjeldahl method, regulated to 1 mgN/ml with saline, and stored in a refrigerator after adding 1/100 of 1 % merthiolate.

4. Immunization of fish

Every 5 days, 10 carps were anesthetized with MS-222 and injected intraperitoneally with the SRBC stroma suspension (0.2 ml/fish). At the same time, 0.4 ml of blood was collected from the dorsal aorta of each fish to investigate the time course of antibody production. The injection of stroma suspension was repeated 6 times, whereas the blood-collecting was repeated 10 times (until 20 days after the last injection).

5. Collection of antiserum and normal serum

Five days after the last injection, two fish which showed high hemolysin titers were anesthetized and ca. 8 ml of blood was withdrawn from each fish using 2 ml tuberculin syringes with a 24 G-needle. The blood was allowed to stand at 30°C for 30 min and 0°C for one hour, the clot was removed by centrifuging at 3000 rpm for 5 min and the antiserum obtained as supernatant was diluted 1 : 2 with GVB^{2+}, heat inactivated (as described below) and stored at −35°C.

Normal sera as a source of complement were obtained in a similar manner uninjected fishes, and stored at -35°C without dilut were stable for at least one month.

Heat inactivation of complement

The anti-serum obtained from a fish on the 15th day of immunization was diluted 1 : 30 with GVB^{2+}, 0.5 ml aliquot was heated at different temperatures

(1×10⁹/ml) of SRBC suspe 0.9 ml o f GVB^{2+}

SRBC. of

OD_{541}

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7. Preparation of sensitized sheep red blood cells (EA)

Sheep red blood cells were washed with GVB(f) and suspended at a concentration of 1×10^9/ml in the same buffer. Antiserum was diluted 1 : 100 to 1 : 200 with EDTA•GVB (the dilution for the optimum sensitization was determined by usual way). Equal volumes of SRBC suspension and of antiserum dilution were mixed and incubated at 30°C for 30 min with occasional shakes. The mixture was cooled and centrifuged at 2000 rpm for 5 min and the precipitate (EA) was washed twice with GVB(f), and finally suspended in GGVB to give a cell density of 5×10^8/ml and stored at 0°C. Just before use EA was washed and resuspended in GVB^{2+} or GVB(f).

8. Optimum conditions for assaying hemolytic complement titer

The immune hemolysis reaction was carried out by reducing the total volume of Mayer's method (5.0 x 10^8 cells/7.5 ml) to one-fifth (1 x 10^8 cells/1.5 ml)

1) reaction temperature

The effect of temperature on hemolytic reaction was investigated as follows : the mixture of 0.2 ml of EA (5 x 10^8/ml), 0.8 ml of GVB^{2+} and 0.5 ml of complement diluted with GVB^{2+} to give 70 to 80 % hemolysis (1 : 20 to 1 : 30) was incubated at different temperatures ranging from 10" to 37°C for 60 min, followed by cooling and centrifuging at 3000 rpm fo OD_{541} temperature was determined from supernatant.

2) pH

The pH of GVB^{2+} was readjusted to various pH's ranging from 5.8 to 9.0 by adding 1 N HCl or 1 N NaOH, and hemolytic react pH. The reaction mixtures were incubated at 30°C for 60min and centrifuged, and hemolysis rate at each pH was calculated from OD_{541} of the supernatant.

3) concentrations of Ca^{2+} and Mg^{2+}

In order to remove as much Ca^{2+} and Mg^{2+} as possible, 0.5 ml of complement was passed through an Amberlite IRC-50 (3 ml), then 2.8 ml of GVB(f) was added to the column, the first eluate (0.3 ml) being discarded and the next eluate (2.5 ml) being collected. This eluate was further diluted 1 : 3 with GVB(f).

Aliquot (0.5 ml) of the above cation free eluate (complement) and 10 μl aliquots of $CaCl_2$ solution and $MgCl_2$ solution were added to 0.2 ml of EA, and then the total volume was controlled to 1.5 ml with GVB(f). The mixtures were incubated at 30°C for 60 min. After cooling and centrifuging, the hemolysis rate was calculated from OD_{541} in the supernatant. In refernce to Mayer's method, the optimum concentration of Ca^{2+} was examined in the presence of 0.5 mM Mg^{2+}, whereas that of Mg^{2+} was investigated in the presence of 0.15 mM Ca^{2+}. The concentrations of Ca^{2+} and Mg^{2+} were measured by use of atomic absorption spectophotometer (Nippon Jarrell-Ash Co., AA-500).

9. Assay of hemolytic complement titer (CH_{50})

Diluted complement (1 : 20 to 1 : 40 with GVB^{2+}; 0.4, 0.6, 0.8 ml) was added to 0.2 ml of EA (5 x 10^8/ml) and the total volume was adjusted to 1.5 ml with GVB^{2+}. The mixture was incubated at 30°C for 60 min, and hemolysis rate,

y, was calculated from OD_{541} of the supernatant.

The values of $y/(1-y)$ were plotted on a log-log scaled graph paper (X-axis was $y/(1-y)$ and Y-axis was ml of diluted complement), the volume (ml) which gives 50 % hemolysis $[y/(1-y)=1]$ was read on the graph and complement titer (CH_{50} unit/ml) was calculated *as* follows:

$$\text{complement titer } (CH_{50} \text{ unit/ml}) = \frac{\text{dilution of complement}}{\text{ml of 50 \% hemolysis}} \times \frac{1}{5}$$

10. Assay of hemolysin titer

Antiserum (carp anti-SRBC hemolysin) was diluted with GVB^{2+} by a two-fold dilution method (1 : 50, 1 : 100, 1 : 200...) at a volume of 0.5 ml in small test tubes, then 0.1 ml of SRBC suspension (1 x 10^8/ml), 0.4 ml of GVB^{2+} and 0.5 ml of complement diluted 1 : 20 to 1 : 40 were successively added to the tubes. The mixture was incubated at 30°C for 60 min, centrifuged at 3000 rpm for 5 min, and hemolysis rate (y) was calculated from OD_{541} in the supernatant. The value of y was plotted on a semi-logarithmic graph paper (Y-axis was y and logarithmic X-axis was the dilution of hemolysin). Hemolysin titer, the dilution giving 50 % hemolysis ($y=0.5$), was read on the graph.

RESULTS

1. Antibody production of carp

Ten carps were inoculated with 0.2 ml of SRBC stroma suspension 6 times at 5-day intervals and the time course of their antibody production was followed by assaying hemolysin titers (Fig. 1). The hemolysin titer of each fish began to rise on the 15th day and reached to maximum by the 25th to 35th day of immunization. The injection of immunogen was stopped on the 25th day, but the hemolysin titers of several fishes continued to rise until the 30th day and then gradually decreased till the end of experiment. As shown in Fig. 1, there was little difference in days for the hemolysin titers of each fish to reach to maximum, though the variation of their titers among individual fishes were very large.

2. Heat inactivation of complement

In order to investigate the condition for the heat inactivation of complement which coexists within serum, carp antiserum was heated at various temperatures for 20 min, and the remaining hemolytic activity was assayed by adding defined number of SRBC. As Fig. 2 shows, the inactivation of carp antiserum was successfully accomplished by heating at 50°C for 20 min. Heat inactivated antiserum was stable for at least 3 months at -35°C.

3. Optimum conditions for assaying hemolytic complement titer (CH_{50})

The total reaction volume of Mayer's method was reduced to one-fifth, and the hemolytic activity of carp complement was measured at various temperatures ranging from 10" to 37°C for 60 min. As shown in Fig. 3, the hemo-

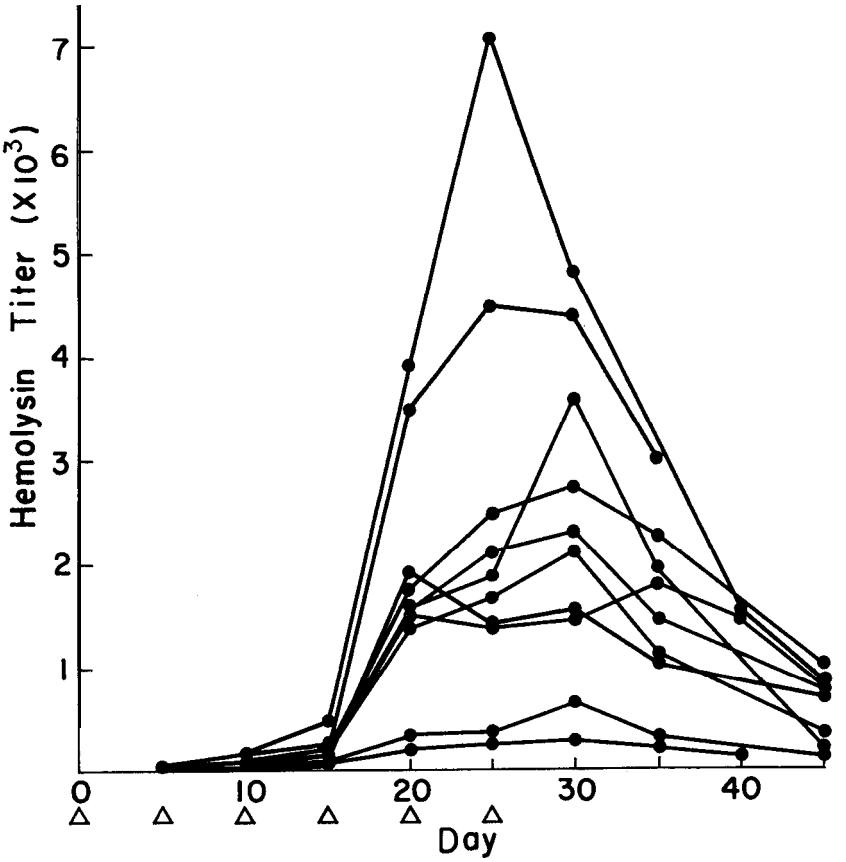


Fig. 1. Antibody productions of ten carps injected with SRBC stroma suspension (1 mgN/ml). The hemolysin titer of each fish was assayed at 5-day intervals until the 45th day. Triangles indicate the days of injection of SRBC stroma suspension (0.2 ml/fish).

lysis rate was maximum at 25°C and maintained high levels of activity in the range of 20" to 30°C, while at 37°C, SRBC were scarcely hemolyzed in contrast to mammals.

As shown in Fig. 4, the hemolytic reaction was also performed at different pH's ranging from 5.8 to 9.0. Consequently, the highest hemolysis rate was observed at pH's from 7.2 to 8.5, but hemolysis rate dropped rapidly at pH's below 7.2.

Fig. 5 a shows the change of hemolysis rate when the Ca^{2+} concentration was altered from 10^{-6} to 10^{-2} M in the presence of 0.5 mM Mg^{2+}, whereas Fig. 5b shows the change of hemolysis rate when Mg^{2+} concentration was altered from 10^{-6} to 10^{-2} M in the presence of 0.15 mM Ca^{2+}. The figures show that hemolytic reaction scarcely proceed at Ca^{2+} or Mg^{2+} concentration lower than

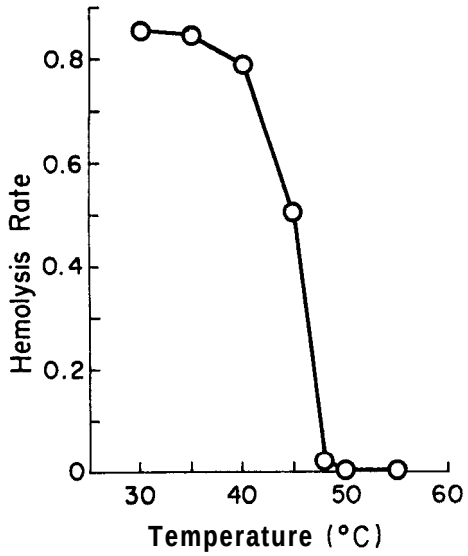


Fig. *2.* Heat inactivation of carp complement. Anti-SRBC carp serum was heated at 30" *to* 50°C for 20 min, and residual hemolytic activity was assayed by adding the defined number of SRBC at 30°C for 60 min.

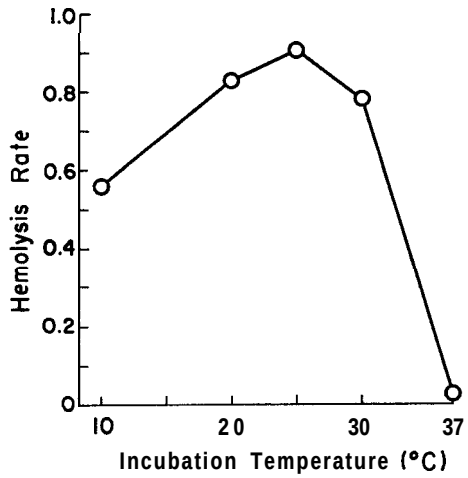


Fig. 3. Effect of incubation temperature on the hemolytic activity of carp complement. The reaction was performed at pH 7.4 for 60 min.

10^{-6} M. This indicates that both cations are indispensable for complement activity. The optimum concentrations of Ca^{2+} and Mg^{2+} were lx 10^{-4} to 5 x 10^{-4} M and 5 x 10^{-4} to lx 10^{-3} M, respectively.

In conclusion, Mayer's method is applicable to the titration of fish com-

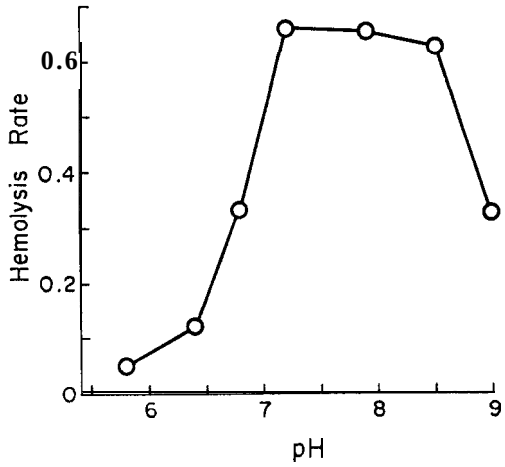
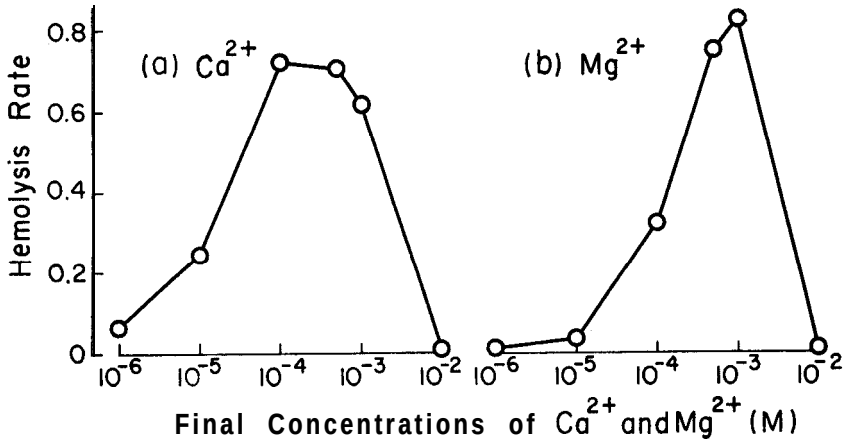


Fig. 4. Effect of pH on the hemolytic activity of carp complement. The reaction was performed at 30°C for 60 min.



Pig. 5. Effects of concentrations of Ca^{2+} and Mg^{2+} on the hemolytic activity of carp complement. The reaction was performed at 30°C for 60 min. (a) Concentration of Ca^{2+} was varied in the presence of 5×10^{-4} M $MgCl_2$. (b) Concentration of Mg^{2+} was varied in the presence of 1.5×10^{-4} M $CaCl_2$.

plement activity by modifying its reaction temperature. As mentioned above, the optimum reaction temperature of carp complement was 25°C. However, the authors adopted 30°C instead of 25°C since the room temperature of our laboratory exceeds 25°C during summer.

4. Stability of complement.

It is generally said that fish complement is more labile than those of mammals. Therefore, an experiment was performed to recognize the stability

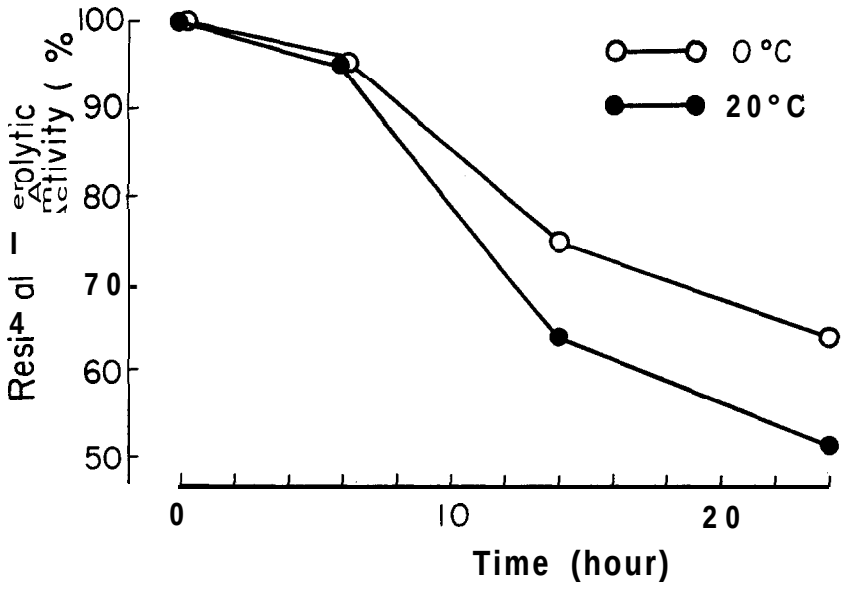


Fig. 6. Stability of carp complement which was allowed to stand at 0°C or 20°C for 24 hours. Residual hemolytic activity was expressed as percentage of the intial one.

of carp serum. The samples frozen at −35°C were thawed at room temperature and then allowed to stand at 0°C or 20°C for 24 hours. Fig. 6 shows that at both temperatures the lowering of complement activities were less than 5 % within 6 hours, and that rapid reduction of activities occurred thereafter, i.e., the samples kept at 0°C showed 36 % decrease in activity at the end of experiment, while those kept at 20°C showed 48 % decrease.

5. **Seasonal variation of complement titers of carp**

Carps, weighing 500-700 g, were captured several times at fish farms located in the south of Fukuoka Prefecture from early in September in 1983 till late in May in 1984. The blood was collected by bloodletting from the caudal peduncles and the hemolytic complement titer (CH_{50}) of the serum was assayed according to the method described above. As seen in Fig. 7, the complement titers gradually decreased as the temperature fell in winter. However, the complement titers began to increase in early spring and reached high values averaging 22.4 CH_{50} units/ml in May.

DISCUSSION

In order to determine the cotnplement activity of carp, we prepared sheep red blood cells (SRBC) sensitized with carp antibody, because fish complements are incompatible with mammalian antibodies. The authors employed SRBC stromata instead of SRBC for immunization of carp, since the former has the

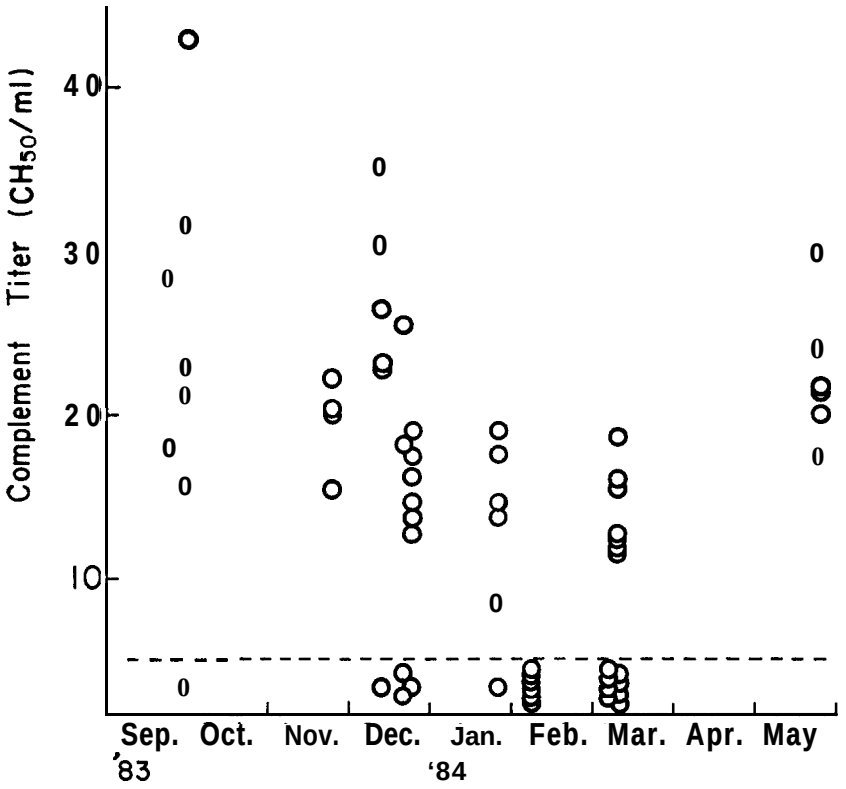


Fig. 7. Seasonal variation of complement titers of carps which were bred in outdoor crawls of fish farms.

advantages of giving low agglutination titer and of yielding antibody suitable for hemolytic reaction. In the preliminary studies, boiled stroma suspension (Nishioka, 1966) was used as immunogen, but it failed to elevate antibody titer. Therefore, in this experiment stroma suspension was injected without boiling.

The carp injected with SRBC stroma suspension successively 6 times at 5-day intervals produced a high levels of hemolysin titers (Fig. 1). On the contrary, the primary immune response of carp was not so high as was expected, i.e., the hemolysin titers of carp injected with the same immunogen only once were lower than 200 throughout the experiment.

It is also demonstrated in this experiment that Mayer's method which was devised for the titration of mammalian complement was applicable to carp complement by modifying the reaction temperature from 37°C to 30°C or below. The optimum hemolytic reaction temperatures reported by several investigators are different among fishes e.g., 28°C for goldfish and elasmobranchs (Legler and Evans, 1967 a, b) ; 30°C for carp, nurse shark, etc. (Day et al., 1970) ; 30°C for rainbow trout and goldfish (Sakai, 1981) ; 25°C for rainbow

trout (Nonaka et al., 1981). The carp complement showed a high hemolytic activity over a wide range of 20" to 30°C in this experiment. This property may be advantageous to poikilothermic animals such as fish.

The hemolytic complement titers of the sera which were allowed to stand at 0°C and 20°C for 24 hours decreased by 36 % and 48 %, respectively. This result was in a marked contrast to that obtained from guinea pig serum which showed 8% decrease at 0°C and 19 % decrease at 20°C in 24 hours (Yano et al., unpublished). Therefore carp serum should be kept at 0°C after thawing and has to be used as soon as possible (within 6 hours).

The hemolytic complement titers of carp were very low in winter. This may be due to the reduction of fish's taking foods because the fishes fed in aquaria (2411°C) in this season showed appreciablly high levels of complement titers.

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