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ESTIMATION OF CLASSICAL PATHWAY OF MOUSE COMPLEMENT ACTIVITY BY USE OF SENSITIZED RABBIT ERYTHROCYTES

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A simple photometric assay was devised for determining classical complement pathway activity in mouse serum using sensitized rabbit erythrocytes as target cells. These cells appeared more sensitive to lysis by mouse complement than sensitized mouse and sheep erythrocytes, most probably by their ability to escape the C3b inactivator system. Advantages of the assay over other techniques are the high sensitivity and the avoidance of the use of radioisotopes. With this test it is possible to get more insight in the complement system of an animal species that has been most widely in use in immunological research.

INTRODUCTION

Mouse serum behaves deficient in the usual photometric complement (C) tests employing sheep erythrocytes (ShRBC) as target cells (Brown, 1943). Although modification to ^{51}Cr-release tests greatly improved the results for mouse serum (Berden et al., 1978), this adaptation did not eliminate the great difference in efficiencies between mouse and, e.g., guinea pig C. However, with rabbit erythrocytes (RaRBC) as target cells no great differences between alternative C pathway activities of mouse and guinea pig serum were measured (Van Dijk et al., 1980a). As alternative C pathway activation by RaRBC is thought to occur by hindrance of the C3b inactivator system (Fearon and Austen, 1977), this system is most probably responsible for the failing lysis of ShRBC by mouse serum, which was already suggested by Borsos and Cooper in 1961.

The relative unsensitiveness to the C3b inactivator system prompted us to study the classical C pathway activity of mouse serum using sensitized RaRBC (RaRBC-A) as target cells. CH50 values thus obtained were compared to similar values from tests using sensitized mouse erythrocytes (MoRBC-A), which are activators of the human alternative C pathway (Kazatchkine et al., 1979; Van Dijk et al., 1980a) and sensitized ShRBC (ShRBC-A). Classical pathway of mouse C activation by RaRBC-A was studied in more depth.

MATERIALS AND METHODS

Animals

Unless otherwise mentioned, male F_1 (BALB/c × Swiss inbred) mice of about 11 weeks of age, bred and maintained in our laboratory, were used as serum, plasma and MoRBC donors. In one experiment female mice were used and in another male mice of the parent BALB/c and Swiss inbred strains and of the C3H/HeJ and C3HeB/FeJ (Jackson Laboratories, Bar Harbor, ME, U.S.A.) (sub)strains.

Serum and plasma preparation

Mice were bled from the retro-orbital venous plexus by means of capillary tubes (Drummond, Broomall, PA, U.S.A.). After the blood was allowed to clot at room temperature for exactly 2 h, serum was separated by centrifugation at 4° C and used immediately.

In one experiment mouse plasma was used obtained by mixing 40 μl of a millipore-sterilized Mg-EGTA stock solution, pH 7.4 (containing 0.5 M $MgSO_4$ and 0.2 M ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid; Van Dijk et al., 1980a), with 0.96 ml fresh mouse blood and subsequent centrifugation. For recalcification 200 μl plasma were diluted 1 : 10 in the test buffer with an admixture of 0.8 mM calcium.

Freshly collected guinea pig serum was stored at −70° C.

Buffers

Five times concentrated veronal-saline buffer, pH 7.4 (VSB-5x), was prepared as described by Mayer (1961).

Veronal-saline buffer, pH 7.4, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VSB^{2+}) was prepared with VSB-5x and a 1000 × concentrated stock solution of calcium and magnesium chloride (Mayer, 1961).

Veronal-buffered saline, pH 7.4, containing 20 mM Mg^{2+} and 8 mM EGTA (EGTA-VB) was prepared by mixing 20 vol of VSB-5x with 4 vol of the Mg-EGTA stock solution as described under *Serum and plasma preparation* and 76 vol of distilled water (Takada et al., 1978).

Veronal-buffered saline, pH 7.4, containing 10 mM ethylenediamine tetraacetic acid and 0.1% gelatin (EDTA-GVB) was also prepared as described by Takada et al. (1978).

Erythrocytes

Freshly obtained mouse blood was diluted 1 : 2 with Alsever's old solution and used as source of MoRBC.

Similarly diluted rabbit and sheep blood preparations were obtained from the National Institute of Public Health (J. Blonk, Bilthoven, The Netherlands) and used as source of RaRBC and ShRBC. RaRBC were regularly used within 5 days and ShRBC within 3 weeks after the blood was collected.

To elute possibly adsorbed serum proteins (Freedman and Massey, 1979)

all erythrocytes were before use washed 3 times with an isotonic solution of sodium iodide (0.16 M) in water (Najjar, 1974; Fyhrquist and Wallenius, 1975).

Anti-erythrocyte sera

Goat antisera against RaRBC and MoRBC were kindly supplied by Dr. R.H. Van Dam and Mr. P.J.S. Van Kooten (Department of Immunology, Veterinary Faculty, State University of Utrecht, The Netherlands). They were prepared by subcutaneous priming and, after an interval of 2 weeks, boosting (weekly for 3 or 4 times) with 10^{10} red blood cells (RBC). Two weeks after the last booster the animals were bled for serum.

Rabbit anti-ShRBC serum (haemolytic amboceptor) was obtained from the National Institute of Public Health (Bilthoven, The Netherlands).

Preparation of sensitized erythrocytes

The concentration of washed RBC was photometrically adjusted to 3×10^8 cells/ml VSB^{2+}. The cells were 1 : 2 diluted with an appropriate dilution of heat-inactivated (30 min, 56°C) antiserum (A) in VSB^{2+} and incubated under magnetic stirring for 10 min at room temperature. The antiserum dilutions were: for RaRBC 1 : 10, for MoRBC 1 : 5 and for ShRBC 1 : 400, respectively. After incubation the cells were washed once and resuspended in VSB^{2+} to a final concentration of 1.5×10^8 cells/ml. Sensitized RBC thus prepared were further designated as RBC-A.

Haemolytic assays

In the original assay, analogous to that for mouse alternative C pathway determination (Van Dijk et al., 1980a), 200 µl of mouse (or guinea pig) serum serially diluted in VSB^{2+} were mixed and subsequently incubated for 30 min at 37°C with 100 µl of RaRBC-A, MoRBC-A or ShRBC-A (1.5×10^8 cells/ml). To stop the reaction 2.2 ml of ice-cold VSB^{2+} were added, after which the cells were spun down and the E_{412} of the supernatant was measured. Corresponding dilutions of heat-inactivated serum similarly incubated with sensitized erythrocytes served as 0% lysis controls. The percentage of haemolysis (Y) was calculated against a water-lysed 100% lysis control and a buffer control for 0% lysis using the formula:

$$Y = \frac{E_{412}\,(\text{fresh serum}) - E_{412}\,(30\text{ min }56^\circ\text{C serum})}{E_{412}\,(\text{water}) - E_{412}\,(\text{buffer})} \times 100\% .$$

The E_{412} (water) for RaRBC-A was about 1.23, while the E_{412} (buffer) was regularly within the range of 0.070—0.130.

The amount of serum used (in µl) logarithmically plotted against log (Y/100 − Y), following the Van Krogh equation (Mayer, 1961), yielded a linear curve. The CH50 value was read from the plot at the value log (Y/100 − Y) = 0.

Similar tests were performed using EGTA-VB as test buffer instead of

VSB^{2+}. The thus obtained log (Y/100 – Y) values were used to determine alternative C pathway activities (AP50 values). No lysis occurred when EDTA-GVB was used as test buffer.

The definitive assay was performed with a similar incubation volume and number of RaRBC-A, but with an incubation temperature of 30° C and an incubation time of 60 min.

Cobra venom factor treatment of serum donors

Cobra venom factor (CoF), purified according to Ballow and Cochrane (1969), was kindly provided by Dr. W. Seinen (Pathology Institute, Veterinary Faculty, State University of Utrecht, The Netherlands). Mice were intraperitoneally injected with 2 × 2 units of CoF with an interval of 8 h. Sixteen hours after the last injection the mice were bled for serum.

Treatments of mouse serum

Zymosan treatment was performed by incubating fresh undiluted mouse serum with 1% of sonicated zymosan during 60 min at 37° C (Mayer, 1961). After centrifugation the supernatant was used.

Sensitivity of the mouse classical C pathway to storage was tested by freezing fresh mouse serum at —70° C and thawing it 24 h later of by incubating it for varying periods at +20, 37, 48 and 56° C. Immediately after these procedures the CH50 activities were determined.

Inhibition experiments

ε-Aminocaproic acid to a final concentration of 50 mM (Soter et al., 1975), heparin (15 U/ml; Jaques, 1979), L-lysine (20 mM; Takada et al., 1978) and suramin (0.02%, Fong and Good, 1972) were used as known inhibitors of the classical C pathway.

Fractionation of the anti-RaRBC serum

In one experiment the goat anti-RaRBC serum was fractionated by affinity chromatography over a protein A-Sepharose CL4B column (Pharmacia, Uppsala, Sweden) and by ultrafiltration (Amicon, Lexington, MA, U.S.A.) using a filter with an exclusion limit of 300,000 d. The material adsorbed to the column was eluted with a buffer containing 22 g citric acid, 10 g glycine and 9 g sodium chloride per liter (glycine-citric acid buffer, pH 2.8). After dialysis and concentration by lyophilization the fractions were dissolved to their original volumes and tested for their 50% lysis concentration (LC50, Van Dijk and Bloksma, 1977) which was determined in the original haemolytic assay using an excess of mouse C (1 : 20 diluted fresh mouse serum).

Statistical analysis

Results were expressed as the arithmetic mean of N duplicate values. The standard error of the mean (S.E.M.) was given for analysis of significance.

RESULTS

Efficiency of different sensitized erythrocytes to activate the murine C system

The CH50 and AP50 values of mouse serum were determined using VSB^{2+} and EGTA-VB as test buffers and sensitized RaRBC, MoRBC and ShRBC as target cells (Fig. 1). RaRBC-A appeared the most efficient activators of the classical pathway of mouse C. Mouse classical C pathway activity as determined with RaRBC even exceeded that of guinea pigs by 40% (data not shown). Sensitized RaRBC were also lysed in EGTA-VB indicating alternative pathway activation. For this phenomenon, however, 3.5 times higher serum concentrations were required. With similar restrictions alternative C pathway activation was observed when sensitized MoRBC were used as target cells. When non-sensitized RaRBC were used classical and alternative pathway activation did also occur, but in this case 4.5 respectively 3 times higher serum concentrations were needed than when sensitized RaRBC were used.

Depletion and inhibition experiments

Serum of CoF-treated mice, zymosan-treated normal mouse serum, or normal mouse serum with or without an inhibitor of classical C pathway activation were tested for CH50 activity with RaRBC-A as indicator cells. Both CoF and zymosan treatment resulted in a complete depletion of the classical C pathway, while ϵ-aminocaproic acid, heparin, L-lysine and suramin, at least in the doses tested, inhibited classical C pathway-mediated haemolysis by 40—60% (Table 1).

Fractionation of the sensitizing serum

The goat serum used for the preparation of RaRBC-A was fractionated

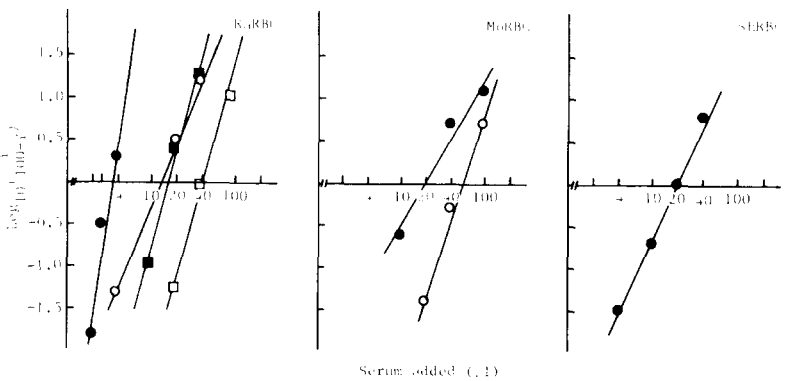

Fig. 1. Classical and alternative C pathway mediated lysis of sensitized erythrocytes (○) and non-sensitized rabbit erythrocytes (□). The closed symbols represent haemolysis in veronal buffer with Ca^{2+} and Mg^{2+}-ions (VSB^{2+}), the open symbols indicate haemolysis in EGTA-VB.

TABLE 1

Effect of C-depleting and inhibitory substances on the CH50 value of normal mouse serum as measured with sensitized RaRBC. For these experiments the original assay was used.

	Inhibition of CH50 activity (%)
Normal mouse serum	0
C-depleting agents	
CoF	>99.9
Zymosan	>99.9
C-inhibitory substances	
ϵ-Aminocaproic acid (50 mM)	49.2
Heparin (15 U/ml)	50.1
L-Lysine (20 mM)	43.7
Suramin (0.02%)	60.3

over a Sepharose-protein A column and by ultrafiltration. The original antiserum and the 4 fractions obtained were tested for LC50 activity. The effluent of the protein A column and the >300,000 d fraction obtained by ultrafiltration were the fractions which exhibited significant haemolysin activity (Table 2).

Optimal conditions for mouse serum CH50 value determination with RaRBC-A as target cells

To determine the optimal conditions for the assay of mouse classical C pathway activity the incubation temperature, time and volume of the original method according to Takada et al. (1978) and Van Dijk et al. (1980a) were varied and moreover the cell numbers per test tube. Incubation temperatures of 22–30°C (Fig. 2) and a time of 60 min (Fig. 3) were found to be optimal. Reduction of the incubation volume and the number of target cells increased further the sensitivity of the assay (Figs. 4 and 5). For practical

TABLE 2

Haemolysin activity of fractionated goat anti-RaRBC serum. For testing of LC50 activity the test conditions of the original assay were used.

Fraction	LC50 activity (U/ml)
Unfractionated serum	1260
Effluent protein A column	1780
Adsorbate protein A column	126
>300,000 d (ultrafiltration)	1580
<300,000 d (ultrafiltration)	200

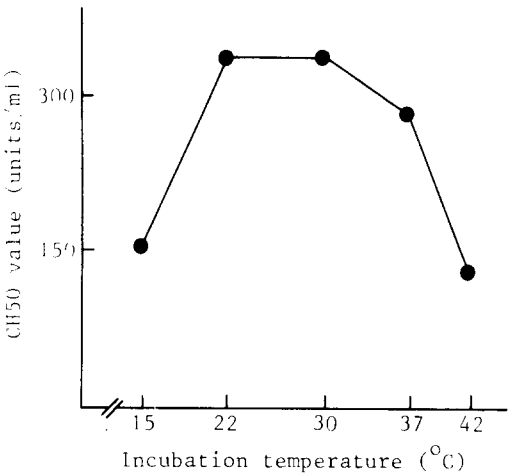

Fig. 2. The influence of varying the incubation temperature on the CH50 value of mouse serum as determined with sensitized RaRBC as target cells. The assay was performed in a volume of 300 μl, with a number of 1.5×10^7 target cells and with an incubation time of 30 min.

and statistical reasons, however, the original test conditions of 300 μl and 1.5×10^7 RaRBC-A per tube were maintained in further experiments.

Effect of storage of mouse serum at different temperatures on classical C pathway activity

The stability of the classical pathway of mouse C to freezing (−70° C) and thawing and incubation at temperatures >0° C was investigated. Mouse serum C appeared readily stable on freezing and thawing (Fig. 5). The curves for storage at +20, 37 and 48° C showed an intermediate minimum and maxi-

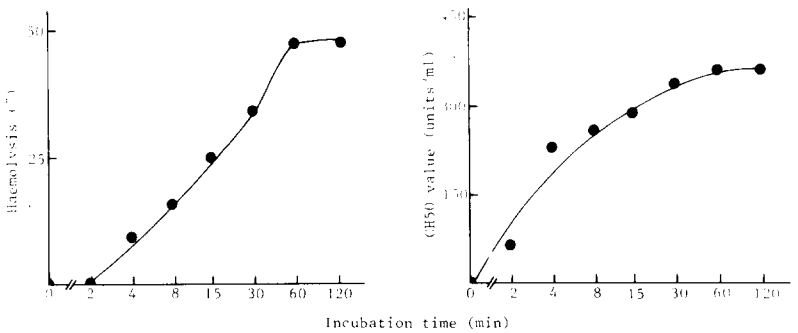

Fig. 3. Effect of varying the incubation time on the development of the lysis of sensitized RaRBC by one mouse CH50 unit and on the CH50 level of fresh mouse serum. The incubation temperature used was 30°C.

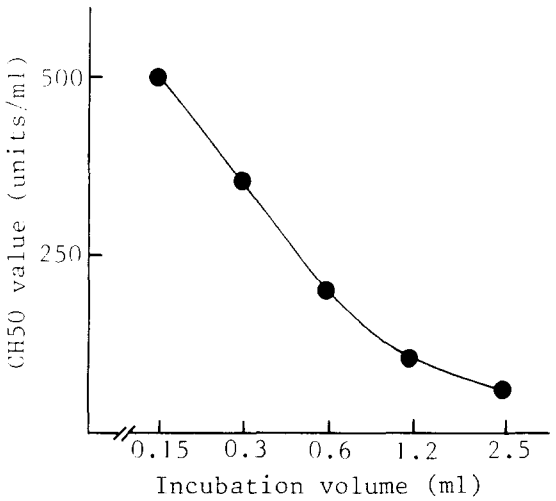

Fig. 4. Effect of reducing the incubation volume on the CH50 value of mouse serum as determined with sensitized RaRBC as target cells. The other test conditions were as in the definitive assay.

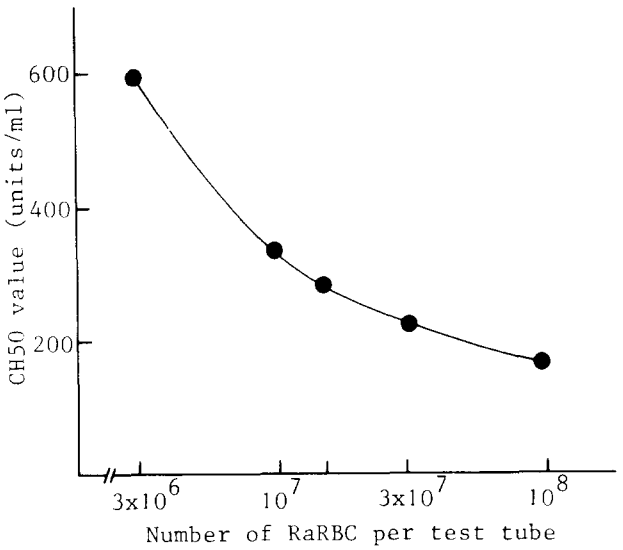

Fig. 5. The relation between the target cell number and the height of the CH50 value recorded for mouse serum. The other test conditions that were employed were those of the definitive assay.

TABLE 3

Differences in CH50 activities.

Mouse (sub)strain	CH50 value (U/ml)			
	Serum		Recalcified EGTA plasma	
	Males	Females	Males	Females
F_1 (BALB/c × Swiss inbred)	338 ± 19 [a] (N = 10)	151	120	<3.5
BALB/c	303	[b]		
Swiss inbred	106			
C3H/HeJ	204 ± 22 (N = 4)			
C3HeB/FeJ	216 ± 14 (N = 4)			

[a] Mean ± S.E.M.
[b] The open places indicate that the values were not determined.

mum. Storage for 4 min at 56°C, 60 min at 48°C, 4 h at 37°C and 24 h at 20°C resulted in complete inactivation of the classical pathway of mouse C.

Comparison of CH50 activities

The CH50 activities of serum and (EGTA) plasma of male and female F_1 (BALB/c × Swiss inbred) mice were determined and compared to the CH50 activities of sera of male BALB/c, Swiss inbred, C3H/HeJ and C3HeB/FeJ mice. Serum CH50 values were substantially higher than values of recalcified EGTA-plasma, while serum and plasma CH50 values of male animals were superior to those of females (Table 3). The CH50 values of F_1 (BALB/c × Swiss inbred) and BALB/c mouse sera were higher than those of Swiss serum. No significant differences between the intermediate serum CH50 vaues of C3H/HeJ and C3HeB/FeJ mice were observed.

DISCUSSION

Our results show that sensitized RaRBC are potent activators of the murine C system (Fig. 1). Using these target cells in a test system derived from an assay for the mouse alternative C pathway (Van Dijk et al., 1980a), mouse serum CH50 values even exceeded the value for guinea pig serum. This suggests that the poor lysis of sensitized ShRBC by mouse C is rather an artefact than that it would have biological significance. The high sialic acid content of ShRBC membranes, functioning as a kind of acceptor site for the C3b inactivator system (Kazatchkine et al., 1979; Ranken and Linscott, 1979), might be responsible for the low C activity observed for mouse serum

using those target cells (Brown, 1943). The sensitivity to EDTA and to heating at 56°C and depletion and inhibition experiments (Table 1) indicated that lysis of sensitized RaRBC by mouse serum involves real classical C pathway activity; CoF and zymosan, both depleting C3, exhausted the lytic activity of mouse serum, while ϵ-aminocaproic acid and L-lysine which inhibit C1 (Soter et al., 1975; Takada et al., 1978) and heparin with a similar activity (Jaques, 1979) moreover potentiating C1 inhibitor (Rent et al., 1976) and suramin which blocks almost the complete C cascade (Fong and Good, 1972) were inhibitory. Contribution of the alternative C pathway to the eventual lysis of sensitized RaRBC by mouse serum could, however, not be excluded, although alternative pathway activation per se (in EGTA-VB; Fig. 1) occurred at higher serum concentrations. In contrast to the lysis of sensitized ShRBC by mouse serum, which seems to be governed by IgG antibodies (Winn, 1965; Berden et al., 1978), lysis of sensitized RaRBC depends on antibodies with a molecular weight greater than 300,000 Daltons and with no affinity for protein A (Table 2), thus most probably on IgM.

The optimal conditions for assaying the murine classical C pathway with sensitized RaRBC as target cells (Figs. 2—4) were determined to be: a target cell number of $1—1.5 \times 10^7$, an incubation volume of 300 μl, an incubation temperature of 22—30°C and an incubation time of 60 min. For the assay thus performed only a small volume of mouse serum is needed, i.e., about 60 μl for duplicate tests, so that is applicable to serum samples of individual animals. The 60 μl include duplicate tests with heat-inactivated serum, which serve as controls for the regularly observed autolysis of mouse serum. This control was preferred by us above the EDTA control as suggested by Lachmann and Hobart (1978), as RaRBC are rather unstable in an EDTA buffer.

With the assay devised it was found that the lysis of sensitized RaRBC by the classical pathway (Fig. 3) develops quickly in comparison to alternative pathway-mediated lysis of non-sensitized RaRBC (Van Dijk et al., 1980a). Further it was observed that mouse C is not that unstable on storage as generally considered (Fig. 6; Borsos and Cooper, 1961). Relatively low CH50 values for female animals (Table 3) were as recorded by many others (a.o. Terry et al., 1964). The poor CH50 activity registered for mouse plasma (Table 3) corresponds to the low alternative pathway activity (Van Dijk et al., 1980a) and might be indicative for the generation of C activity during clotting. A similar suggestion was done with respect to generation of factor $\bar{D}$ (Davis et al., 1978). The equality of serum CH50 activities for C3H/HeJ and control C3HeB/FeJ mice is in disagreement with results of Hoffmann (1978), who used sensitized ShRBC as target cells. It is, however, in line with our hypothesis, that C3H/HeJ mice might have an aberrant C3b inactivator system (Van Dijk et al., 1980b).

In conclusion, the assay developed using sensitized RaRBC as target cells is very useful for measuring murine classical C pathway activity. Advantages over other techniques are a higher sensitivity (Rosenberg and Tachibana, 1962) and the non-involvement of radioactive materials (Andrews and Theofilo-

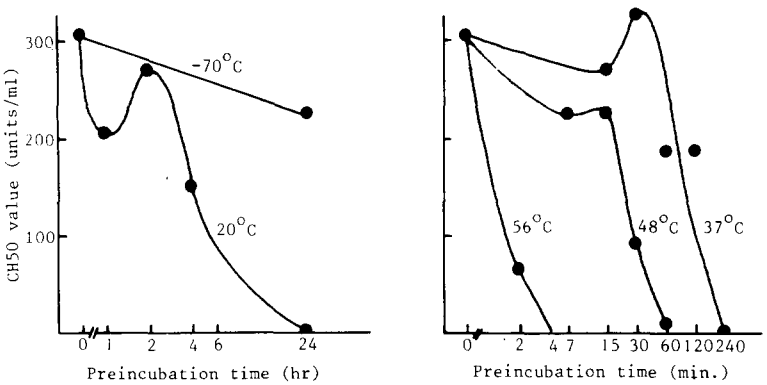

Fig. 6. Sensitivity of mouse classical C pathway activity to storage at different temperatures.

poulos, 1978; Berden et al., 1978). The method offers a simple way of measuring serum C activity in an animal species, the immune system of which has been most intensively studied.

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