

CONTRIBUTION TO THE STUDY OF IMMUNE HEMOLYSIS BY TOAD COMPLEMENT

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EA (sheep erythrocytes carrying rabbit antibody) are lysed by toad complement under optimal conditions which include a low concentration of cells ($1.54 \times 10^8/ml$), a low temperature of incubation ($30^{\circ}C$) and the same amounts of Ca^{++} and Mg^{++} as required for the titration of guinea-pig complement.

Kinetic studies of the role of cations mentioned above in immune lysis by toad C have disclosed a fundamental difference as compared to guinea-pig C. In a limited complement system, the lysis by amphibian C is completely blocked by EDTA, even when the chelating agent is added as late as 15 minutes after zero-time. Inhibition by EGTA is only partial and the findings suggest that Mg^{++} is required not only at the beginning, but also at late stages of the lytic process. It has been speculated that the activation of amphibian complement proceeds mainly by the alternative pathway.

There are suggestive evidences that complement (C) and immunoglobulins have a parallel development during evolution, attaining the highest degree of complexity and efficacy in warm-blooded vertebrates, particularly in mammals, in which the complement system comprise at least 11 protein components, not including inactivators and inhibitors, nor the factors involved in the activation by the alternative pathway.

Both in mice and in man, the genes controlling the cell interactions that lead to the response to specific antigens (Ir genes) and the genes governing the synthesis of certain components of the complement system (C4, C2, factor B) are located in neighbouring regions of the same chromosome.

Complement studies in the sera of reptiles, amphibians and fishes are, however, scarce and were not performed with adequate methodology, being therefore unable to provide reliable data for comparing the composition and functioning of the system in warm and cold blooded vertebrates.

As far as amphibians are concerned, Cushing has shown in 1945 that, unlike guinea-pig complement, the complement of the frog (*Rana catesbiana*) acts on sheep erythrocytes sensitized with rabbit antibody (EA) almost as rapidly at 16° as at 37°, yielding identical titers (around 1:8) after 1 hour of incubation.

Both frog and guinea-pig sera can be inactivated by similar techniques exhibiting C1, C2, C4 and "classical" C3. Also, in *Bufo*, *Rana* and *Necturus*, Legher & Evans have shown (1966) that complement can be inactivated by hydrazine, by carrageenin, by heating and by chelating agents.

Recent data of Day et al (1970) with bacterial polysaccharides point out the importance of the alternative pathway in the activation of the complement system in lower fishes, amphibians, reptiles and birds.

The present paper contributes to the study of toad complement (*Bufo paracnemis*, *Bufo ictericus*) and provides reliable data for a valid comparison with the guinea-pig complement system.

MATERIAL AND METHODS

Diluents

Stock Solution. A five times isotonic stock solution was prepared by dissolving 85 g NaCl plus 3.75 g Na-5.5-diethyl barbiturate in about 1400 ml distilled water; 5.75 g 5.5-diethyl barbituric acid are separately dissolved in 500 ml of hot distilled water.

The two solutions are mixed after cooling at room temperature, pH is adjusted 7.4. and volume is made up to 2000 ml.

GVB 200 ml of stock solution are mixed with 1 g of gelatin previously dissolved in a small volume of hot distilled water and volume is made up to exactly 1000 ml in a volumetric flask: ($\mu = 0.147$).

GVB⁺⁺. Prepared as above by adding 1 ml of a solution containing 0.15M CaCl₂ and 1.0 M MgCl₂ before completing the volume to 1000 ml.

Final concentrations: 0.15mM Ca⁺⁺ and 1 mM Mg⁺⁺.

DGVB⁺⁺. Prepared as GVB⁺⁺, but diluting stock solution 1:10 instead of 1:5 and incorporating glucose to a final concentration of 0.192 M to assure isotonicity ($\mu = 0.074$).

Chelating solutions. For chelating Ca⁺⁺ plus Mg⁺⁺ 0.01 M EDTA (Ethylenediamine sodium tetracetate) in saline was used, and for chelating only Ca⁺⁺ use was made of a solution in saline of EGTA (Ethylene glycol sodium tetracetate) is the final concentration of 1.24×10^{-3} M with added MgCl₂ at the concentration of 3.3×10^{-4} M. In both cases pH was adjusted to 7.2 ± 0.2 .

Reagents

Sheep Erythrocytes. Sheep blood drawn aseptically in modified Alsever's solution (equal volumes) and preserved at 2-5°C was washed 3 times in GVB, resuspended in GVB⁺⁺ and standardized to 10⁹ cells per ml.

Hemolytic Antibody. Rabbit anti-Forssman hemolysin was prepared as indicated by Legher & Evans (1966). Toad hemolysin was obtained by injecting 10^9 cells intraperitoneally on the days 1, 3 and 10 and giving on day 14 a fourth intraperitoneal injection of the same amount of cells suspended in Freund's complete adjuvant. Bleedings were performed 15 days following the last injection. The amount of antibody required for maximal sensitization was determined as described by Kabat & Mayer (1961).

Sensitized Cells. Sensitized cells were dally prepared by slowly pipetting the optimal dilution of antibody into an equal standardized cell suspension with constant swirling of the contents. The mixture was kept at 2–5°C until used.

Complement. Pooled blood obtained from 10-14 toads (*Bufo paracnemis*, *Bufo ictericus*) was allowed to clot at room temperature. The serum was separated from the clot as soon as possible, and immediately stored at –70°C.

Titration of toad complement showed considerable variation for different batches (68-230 CH₅₀ /ml). Under similar experimental conditions, variations of titer for guinea-pig complement were practically nil.

Guinea-pig anti-Toad C₃. Anti-Toad C₃ was produced in guinea-pig by injection of zymosan-toad serum complexes, as described by Mardivey & Mueller-Eberhard (1965) for anti-human B₁ C.

Experimental Procedures

CH₅₀ Assay. Quantitative estimation of the hemolytic activity of toad complement was performed by mixing 1 ml of different dilutions of the serum with 1 ml of EA (1.54 or 5×10^8 /ml). After 90 minutes of incubation at 30°C or 37°C, 5 ml of saline were admixed. Tubes were then centrifuged and supernates were read either at 412 or at 545 nm in order to estimate the percentages of lysis.

Kinetic experiments. Equal volumes of EA 1.54×10^8 /ml and of an adequate dilution of toad complement previously equilibrated to 30°C were quickly mixed (zero-time) and incubated at the same temperature. Complement was previously depleted of ions by gelfiltration through Sephadex G-25.

One ml samples were withdrawn at suitable intervals and pipetted into tubes containing 1.5 ml of the appropriate chelating agent (EDTA or EGTA).

As shown for guinea-pig C, in experiments where C is the limiting factor, the addition of the chelating agent does not stop the lysis completely, unless added during the first few minutes. Therefore, tubes were subject to further incubation at 30°C for 30 minutes in order to detect any lysis due to the piling up of sites equivalent to SCI, 4, 2 prior to the addition of the chelating diluent. After admixing 5 ml of saline, tubes were centrifuged and supernates were read at 412 nm.

Preparation of intermediate complexes

Attempts were made to prepare with toad complement intermediate complexes similar to guinea-pig EACl, 4, 2 or EACl, 4.

Five ml of EA, 5×10^8 /ml, in GVB⁺⁺, were allowed to react strictly at 0°C with 0.5 ml of undiluted toad complement for 10-20 minutes. After treatment, cells were separated by cold centrifugation, washed in ice-cold GVB⁺⁺ and resuspended in 5 ml of the same buffer.

Half of the suspension was immediately cooled to 0°C (Cells A) and the other half was incubated 2 hours at 37°C (Cells B). Both suspensions were adjusted to 1.54×10^8 per ml and used as follows:

a) 1 ml portions of Cells A were mixed with 1.5 ml of guinea-pig serum diluted 1 :37.5 in GVB or DGB-0.01 M EDTA or with different dilutions of toad serum in the same dilluent.

b) Equal volumes of Cells B and of an appropriate dilution of purified guinea-pig C2 were allowed to react at 30°C. Samples of 1 ml were withdrawn at different intervals of time and pipetted into tubes containing 1.5 ml of the same dilutions of toad or guinea-pig serum in GVB or DGVB-0.01 M EDTA as indicated above.

After 90 minutes of incubation at 37°C with occasional agitation, 5 ml of saline were admixed to the tubes containing Cells A and 4 ml to the tubes containing Cells B. In neither case supernates showed any trace of lysis. Identical results were observed in experiments set up with toad serum-EDTA and guinea-pig EACI, 4, 2.

Negative results were also observed in attempts to prepare intermediate complexes with toad complement by substituting rabbit antibody for toad hemolysin in the sensitization of red cells.

Evidence for the formation of an intermediate product by interacting EA with toad complement was obtained, under following experimental conditions.

Volumes of 6 ml EA, 1.54×10^8 /ml, in DGV Ca^{++} , were incubated for 5 minutes at 30°C with toad complement 1 :40 or 1 :60, separated by cold centrifugation, and resuspended in DGVB $^{++}$ to the initial concentration (Suspensions I and II). As a control, EA 1.54×10^8 /ml was mixed with an equal volume of DGVB $^{++}$ and subjected to the same treatment (Suspension III).

Equal volumes of each one of the above suspensions were allowed to react at 30°C with toad complement 1 :60, and 1 ml samples of each mixture were withdrawn at suitable intervals and treated as described in Experimental Procedures.

Results of a representative experiment of this kind are illustrated in Figure 6. The course of lysis was similar for Suspensions I and II, whereas the control cells, not preincubated with toad complement, exhibited a conspicuously different behavior, indicating the presumptive formation of an intermediate complex in cells I and II.

Similar results were obtained by preincubating EA with undiluted toad complement at 0°C for 10 minutes.

Complement inactivation. Classic treatments used for mammal complement as heating, zymosan and ammonia also inactivated toad complement. Mid and end-pieces were also prepared. However recombination of the different fractions did not restore the hemolytic activity of toad complement.

RESULTS

Hemolytic Activity of Toad Complement

The titration of the hemolytic activity of toad complement was investigated with regard to the following variables: a) temperature of incubation; b) ionic strength; c) effect of added Ca^{++} and Mg^{++} ; d) concentration of cells; e) homologous or heterologous anti-sheep red cells.

Results are summarized in Tables I and II.

As seen in Table I, titers obtained by the use of rabbit amboceptor, were increased twofold when tests were set up at 30°C instead of 37°C and slightly lower values were

obtained when the ionic strength of the diluent was lowered from 0.147 (GVB⁺⁺) to 0.074 (DGVB⁺⁺). In the presence of optimal amounts of Ca⁺⁺ and Mg⁺⁺ the titer also increased twice, whereas with the sole addition of Mg⁺⁺ the titer was increased by a factor of 1.5–1.6 and with Ca⁺⁺ alone there was no significant difference of titer. Although the toad serum had been previously depleted of cations it seems that depletion was only partial and that enough Ca⁺⁺ remained to effect the titer by adding Mg⁺⁺ alone.

TABLE I

Hemolytic activity of toad complement as related to temperature of incubation, ionic strength and concentration of sensitized cells

| Ionic Strength | Concentration of EA | <i>CH</i> ₅₀ /ml in tests run at | |
|----------------|----------------------------|---|------|
| | | 30°C | 37°C |
| 0.147 | 1.54 x 10 ⁸ /ml | 190 | 95 |
| | 5.00 x 10 ⁸ /ml | 62 | 30 |
| 0.074 | 1.54 x 10 ⁸ /ml | 160 | 80 |
| | 5.00 x 10 ⁸ /ml | 60 | 34 |

TABLE II

Effect of Ca⁺⁺ and Mg⁺⁺ on the hemolytic activity of toad complement

| Cations added to DGVB | <i>CH</i> ₅₀ /ml |
|--------------------------|-----------------------------|
| None | 33 |
| 0.15 mM Ca ⁺⁺ | 37 |
| 1.00 mM Mg ⁺⁺ | 54 |
| Both | 72 |

By logarithmic plotting of complement dilution against $\frac{y}{1-y}$ the slope of the regression line was higher for the titration run with DGVB⁺⁺ as compared to tests performed with GVB⁺⁺. Consequently the range of hemolytically active dilutions of toad complement was larger at low ionic strength.

Toad complement titrations showed higher titers using rabbit antibody as compared to toad antibody, 230 *CH*₅₀/ml and 41 *CH*₅₀/ml respectively. As seen in Fig. 1, kinetic experiments performed with a limited amount of toad complement chosen as to provide in both systems similar hemolysis rates at the end of the test, indicate a higher velocity of reaction using rabbit antibody.

As a result of these findings, the technique for titrating toad complement was standardized by using DGVB⁺⁺ as buffer-diluent, 30°C for the temperature of incubation, and EA (sheep red cells sensitized with rabbit antibody) at the concentration of 1.54 x 10⁸/ml, as described in Experimental Procedures.

The effect of Ca⁺⁺ and Mg⁺⁺ on the kinetics of immune hemolysis by a limited amount of toad complement

When EA are incubated at 30°C with a limited amount of toad complement and *y* and the percentage of lysis is plotted against time, identical sigmoid curves are obtained

by adding Ca^{++} plus Mg^{++} at zero-time or Mg^{++} alone (Fig. 2, curves I and III). In both cases there is an induction period of 5-7 minutes and lysis proceeds rapidly to attain $y = 0.5$ in 9.5 minutes. After 11 minutes the velocity of lysis decreases, and the curve becomes convex to the abscissa.

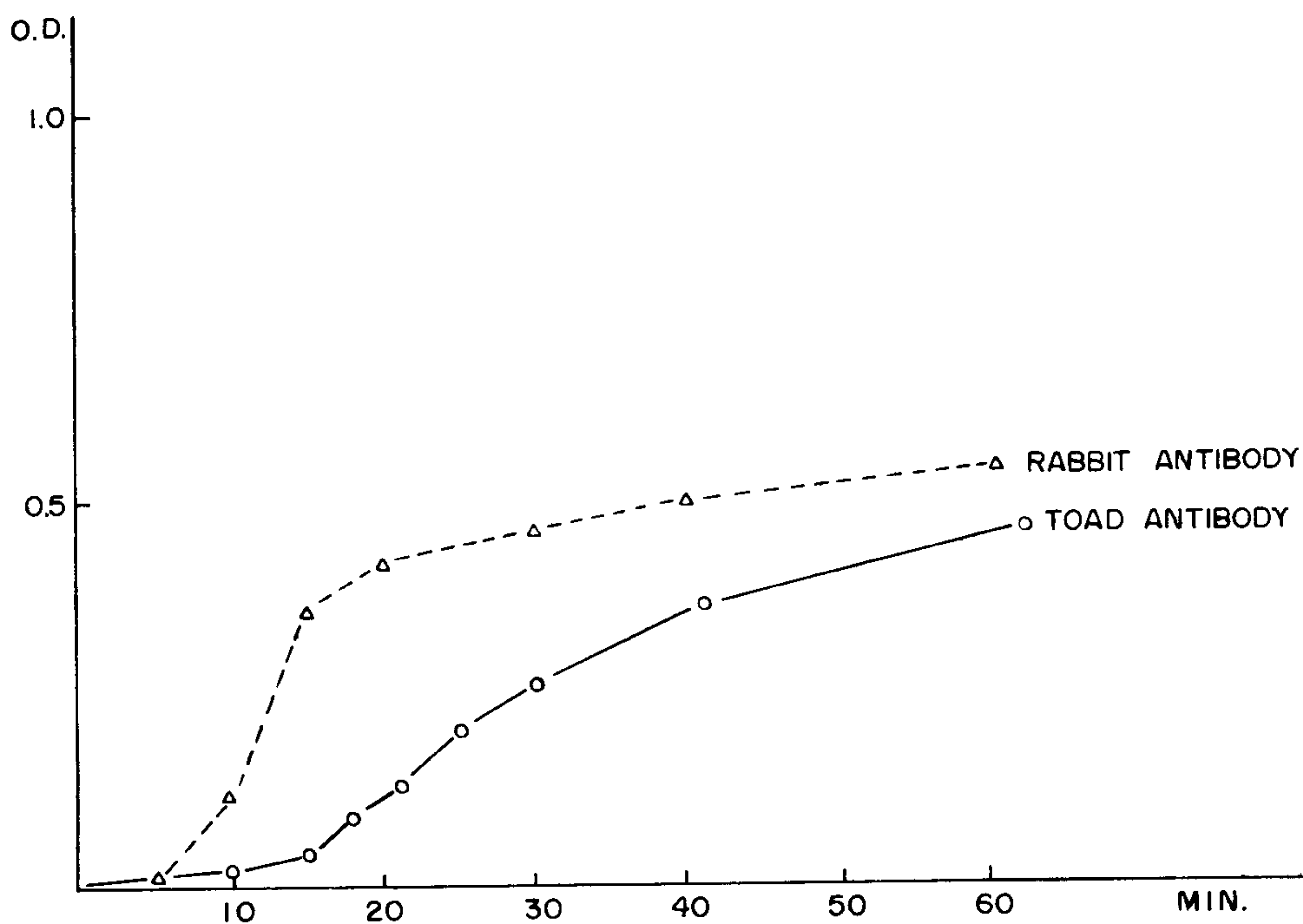


Fig. 1 – Kinetics of immune hemolysis by a limited amount of toad complement by using EA prepared with rabbit or toad antibody.

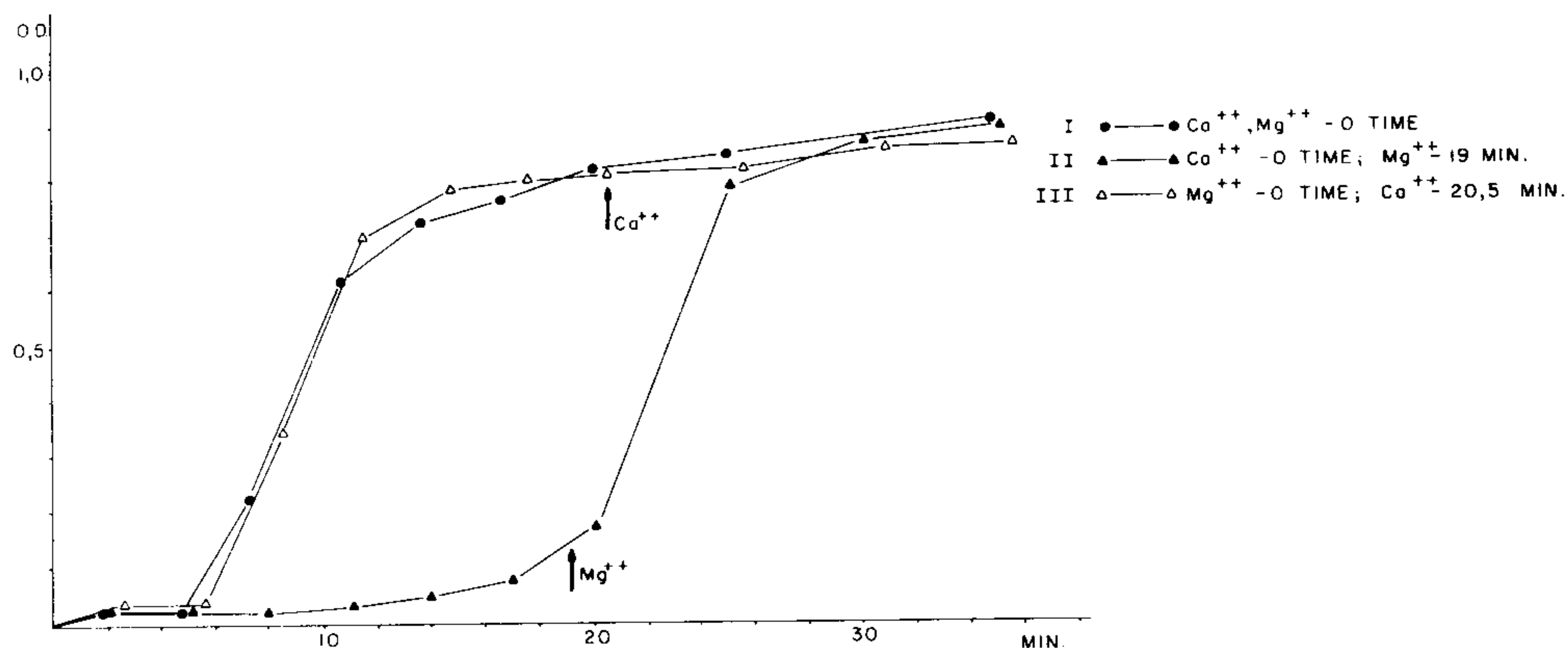


Fig. 2 – The effect of Ca^{++} and Mg^{++} on kinetics of immune hemolysis by a limited amount of toad complement.

If only Ca^{++} is added (Curve II) practically no lysis occurs up to 19 minutes and on the addition of Mg^{++} it reaches $y = 0.5$ at 22.5 minutes.

Experiments were set up to establish the effect of Mg^{++} added at several intervals (0, 5, 10, 15 and 20 minutes) in a system where only Ca^{++} was present (Fig. 3). Curves corresponding to 0 and 5 minutes were similar and the 50% point was attained in 10-11 minutes. However, when the addition of Mg^{++} was postponed to 10, 15 and 20 minutes, $y = 0.5$ was observed after approximately 4 minutes.

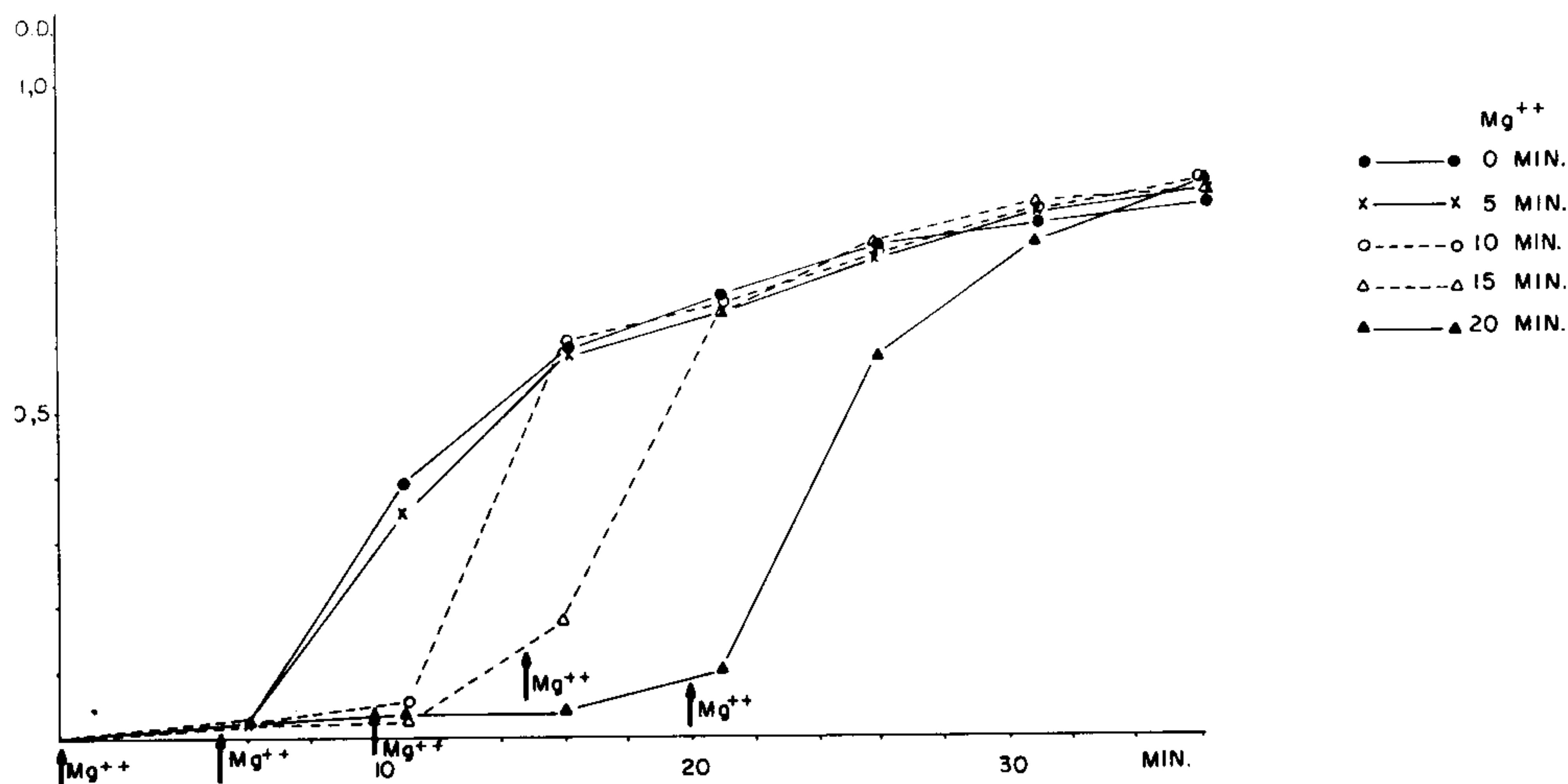


Fig. 3 – Effect of Mg^{++} added at several intervals on the kinetics of immune hemolysis of a system containing a limited amount of toad complement and Ca^{++} .

The effect of Ca^{++} and Mg^{++} was also investigated in kinetic experiments by using EDTA or EGTA as a stop diluent. With EDTA, blocking was complete even when added 15 minutes after zero-time (Fig. 4). Under similar experimental conditions, the effect of EGTA was only partial (Fig. 5). Similar results were obtained by using buffers with high (0.147) or low (0.074) ionic strength.

Electrophoretic conversion of Toad C3

By incubating toad serum with zymosan (25 mg/ml) for 1 hour at $37^{\circ}C$, the immunoelectrophoretic pattern with anti-Toad C3 showed an increase in electrophoretic mobility similar to that observed with mammalian B_1C .

DISCUSSION

The work of previous investigators has shown that the complement of *Amphibia*, in particular frog complement (Cushing, 1945) resembles guinea-pig complement in that the 4 “classical” components can be demonstrated by similar treatments.

Components resembling “classical C3” and C4 could also be demonstrated in frog serum by treatment with zymosan and ammonia but the reactivation of the zymosan fraction either with ammonia-treated or with heat-inactivated serum was found to be irregular and often difficult to achieve.

In our hands, the effect of zymosan and of ammonia on toad complement was not sufficiently clear-cut to allow for definite conclusions. However, evidence for the existence of a component similar to mammalian C3 was given by the conversion of toad β_1C to a faster protein, after treatment of toad serum with zymosan, as verified by

immunoelectrophoresis in the presence of guinea-pig anti-Toad C3 prepared by immunization with toad serum absorbed on zymosan particles.

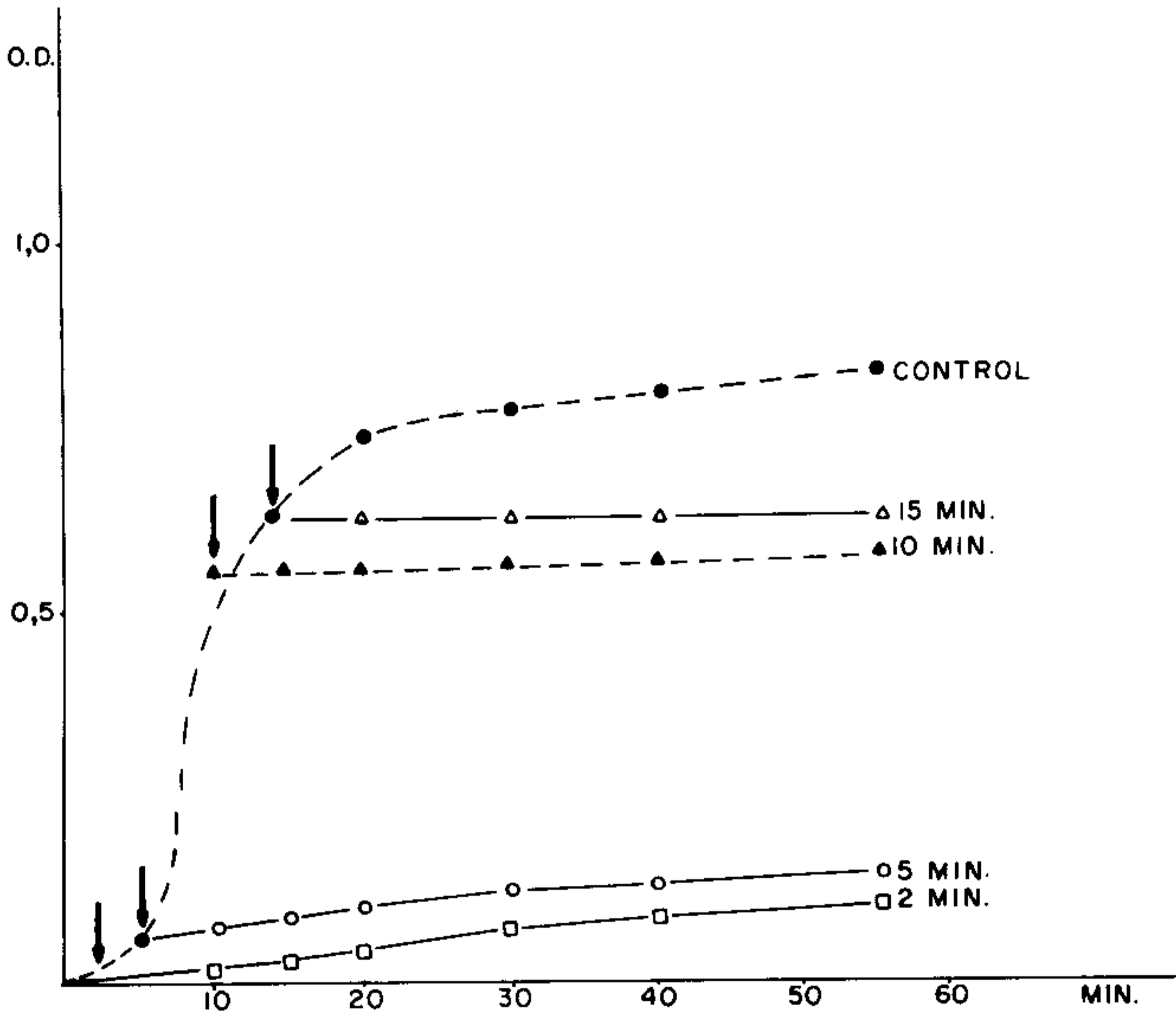


Fig. 4 – Effect of addition of EDTA at several intervals in a limited complement system.

The study of the variables involved in specific hemolysis by toad complement led to the establishment of a standard procedure for titrating toad C in terms of CH_{50} units. This is based on the use of a low concentration of EA, a low temperature of incubation ($30^{\circ}C$) and the same optimal amounts of Ca^{++} and Mg^{++} as used for the titration of guinea-pig C.

However, even by maintaining these optimal conditions, titers of toad C were found to be subject to a large individual variation, perhaps on account of peculiar metabolic changes in the poikilothermic animal.

The kinetic experiments have clearly shown that Ca^{++} and Mg^{++} participate in the mechanism of immune hemolysis by toad complement very much in the same way as with mammalian complement. In both cases, two successive steps can be clearly demonstrated, an initial step depending on Ca^{++} and a second depending on Mg^{++} , but a fundamental difference characterizes the case of the amphibian complement.

Whereas with guinea-pig C the addition of EDTA is only able to stop lysis when added in the first few minutes of the reaction, in the case of toad complement EDTA completely prevents lysis, even when added as late as 15 minutes after zero-time. This

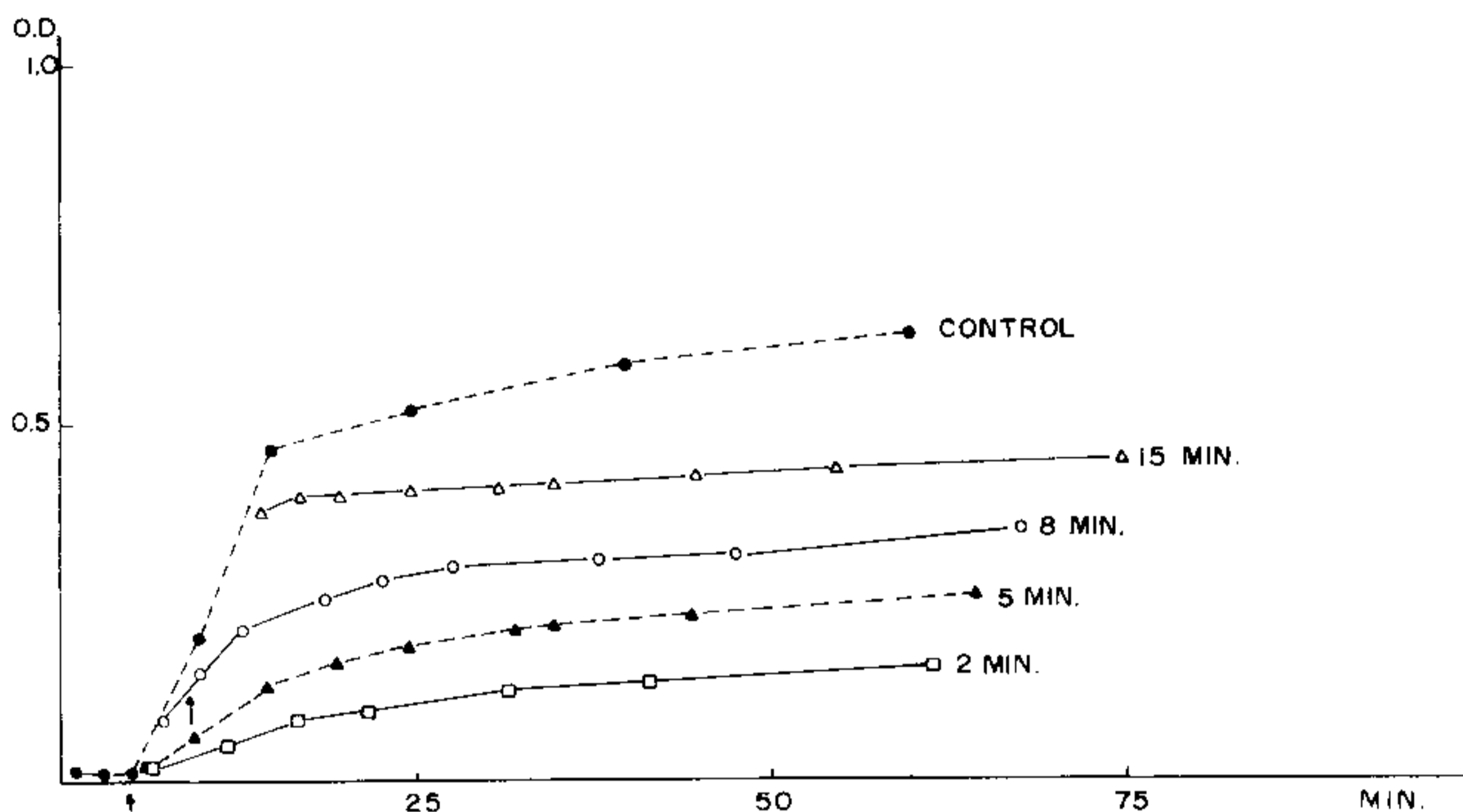


Fig. 5 -- Effect of addition of EGTA at several intervals in a limited complement system.

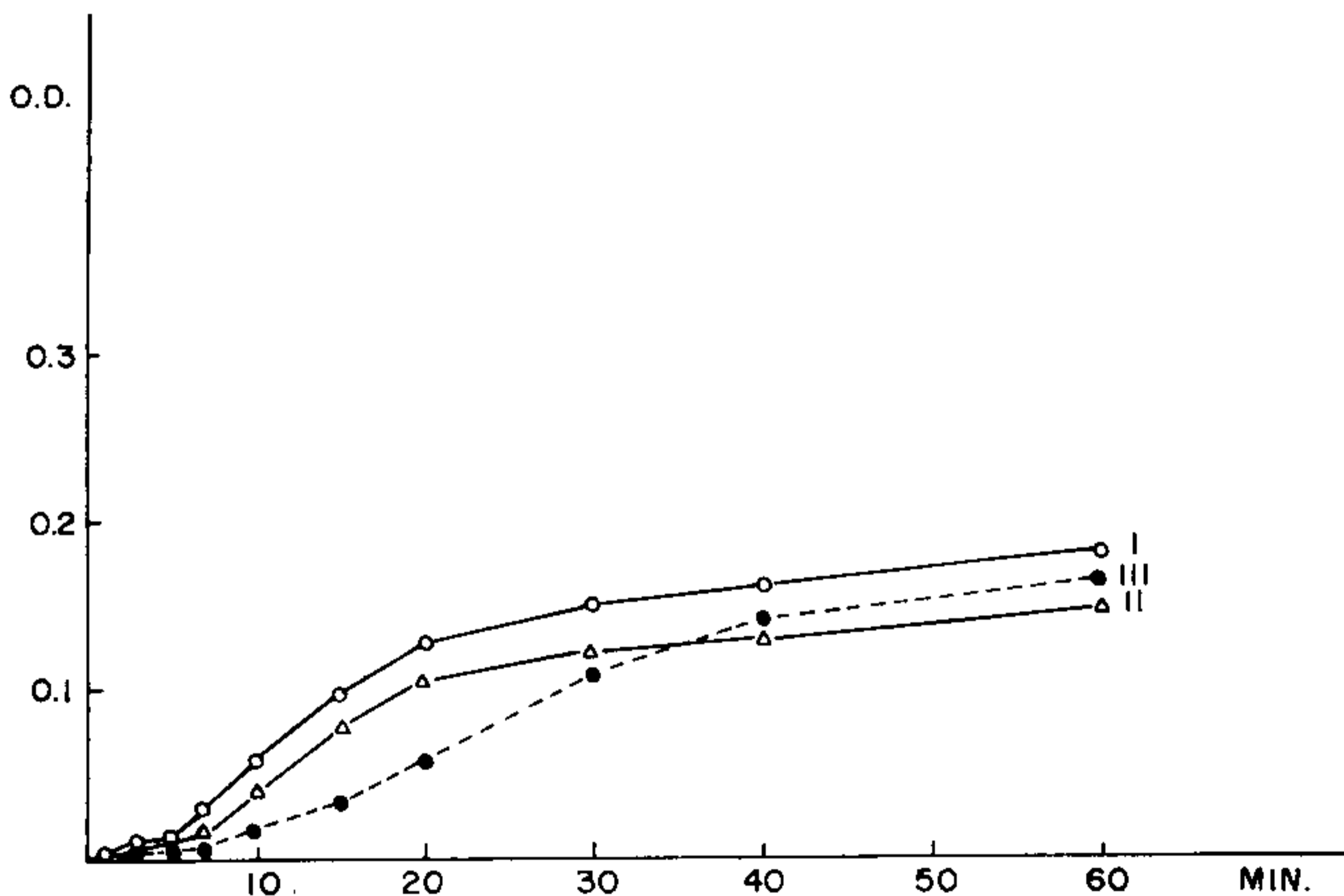


Fig. 6 -- Effect of preincubation of EA with diluted toad complement and Ca⁺⁺.

fact, together with the finding that inhibition by EGTA is only partial, leads to the suggestion that, contrary to what occurs with the mammalian C-systems, Mg⁺⁺ may be required in the amphibian C-system at later stages of the lytic process, after the formation of EAC1, 4, 2. The failure to prepare this intermediate complex under conditions similar to those used with guinea-pig C, may indicate that such a complex, in the toad C-system, is very short lived, on account perhaps of its lability or to a high degree of dissociation.

It would appear that in the case of *Amphibia* and *Reptilia*, in which the immune response is largely non-specific, the activation of complement proceeds mainly by the alternative pathway that requires Mg^{++} for the formation of both C3 – and C5 – convertases (C3b.Bd and C3b₂.Bb). Unstable EAC complexes similar to EAC1, 4, 2 could provide the initial source of C3b and act as a solid phase for the assembling of the “alternative convertases”. On the other hand, the process may also occur in the fluid phase if toad-C3b like cobra-C3b (Hobart & McConnell, 1975) proves to be resistant to splitting by C3-Inactivator.

Further data are obviously required to elucidate this matter which is of utmost importance in comparative immunology.

RESUMO

EA (eritrócitos de carneiro, sensibilizados com anticorpos de coelho) são lisados por complemento de sapo em condições ótimas, que consistem no uso de uma baixa concentração de células ($1.54 \times 10^8/ml$), incubação a baixa temperatura (30°C) e as mesmas quantidades de Ca^{++} e de Mg^{++} , requeridas para a titulação da atividade hemolítica do complemento de cobaia.

Estudos cinéticos do efeito dos cátions mencionados acima na imune-lise produzida pelo complemento de sapo revelam uma diferença fundamental com relação ao complemento de cobaia. Num sistema limitado pela quantidade de complemento, a lise pelo C de anfíbio é totalmente bloqueada por EDTA, mesmo quando a adição do agente quelante é feita após 15 minutos, ao passo que com EGTA o bloqueio é apenas parcial. Os achados experimentais sugerem que Mg^{++} seja requerido não apenas no estágio inicial, mas também em estágios tardios do processo lítico e permitem especular que a ativação do complemento de anfíbio se processa predominantemente pela via alternativa.

REFERENCES

- CHSHING, J.E., 1945. A comparative study of complement, I. The specific inactivation of the components. *J. Immunol.* 50 :61.
- DAY, N.K.; GOOD, R.A.; FINSTAD, J.; JOHANNSEN, R.; PICKERING, R.J. & GEWURZ, H., 1970. Interactions between endotoxic lipopolysaccharides and the complement system in the sera of lower vertebrates. *Proc. Soc. Exp. Biol. Med.* 133 (4) :1397.
- HOBART, M.J. & McCONNELL, J., 1975. The Immune system a course on the molecular and cellular basis of immunity chapter 5. Blackwell Scientific Publ., Oxford.
- KABAT, E.A. & MAYER, M.M. In *Kabat and Mayer's Experimental Immunochemistry*, Second Edition, 1961, Thomas, Springfield, cf. Chapter 4.
- LEGHER, D.W. & EVANS, E.E., 1966. Comparative Immunology. Hemolytic complement in amphibia. *Proc. Soc. Exp. Biol. Med.* 121 (4) :1158.
- MARDIVEY JR., M.R. & MUELLER-EBERHARD, H.J., 1965. Mouse BIC globulin: Production of antiserum and characterization in the complement reaction. *J. Immunol.* 94 :877.