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TECHNICAL MANUSCRIPT 420

A RAPID TURBIDIMETRIC PROCEDURE FOR COMPLEMENT ESTIMATION

William F. Vincent
Earl W. Harris
Sidney Yaverbaum

NOVEMBER 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

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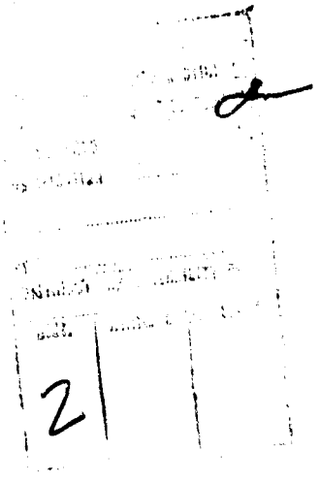

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A RAPID TURBIDIMETRIC PROCEDURE FOR COMPLEMENT ESTIMATION

William F. Vincent

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Physical Defense Division
COMMODITY DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B622401A071

November 1967

ABSTRACT

Complement (C')-mediated hemolysis of sheep erythrocytes followed a first-order kinetics reaction when measured turbidimetrically. A linear standard curve can be constructed from the slopes of the first-order plots obtained with samples of known C' concentration. The standard curve can then be used for the estimation of C' concentration in unknown sera. This direct kinetic procedure is rapid and accurate and employs a minimum of equipment and reagents.

I. INTRODUCTION

The techniques commonly employed for the estimation of complement (C') activity can best be described as static end point titrations, because they are based on the measurement of the extent of hemolysis after the reaction has gone essentially to completion. Plescia, Amiraian, and Heidelberger* demonstrated that C'-mediated hemolysis follows a first-order kinetics reaction and that the time required for a 50% lysis of cells could be used for quantitative estimation of C'. The kinetic method of C' estimation developed by those investigators was preferable to static procedures because the standard curve was linear and the sensitivity was constant over the entire concentration range. The present report describes a kinetic procedure, based on first-order kinetics, that employs the direct turbidimetric estimation of cell lysis to determine the first-order rate constant.

II. MATERIALS AND METHODS

A. SENSITIZED SHEEP ERYTHROCYTES

Sheep blood in modified Alsever's solution** was purchased from Fisher Scientific Co. and stored at 4 C until used. The erythrocytes were collected by centrifuging at 750 x g and washed three times in cold veronal-saline buffer (pH 7.5) containing 2.5 mM Mg^{++} and 0.75 mM Ca^{++}.*** This buffer was employed for all washings and dilutions. The washed erythrocytes were suspended in buffer to a concentration of 5 x 10^8 cells/ml as determined by hemocytometer counts. All suspensions of hemolysin-sensitized cells (EA) were prepared by the procedure of Mayer, Croft, and Gray*** using rabbit anti-sheep hemolysin (Difco) diluted 1:80 except when otherwise noted. An equal volume of diluted hemolysin was added to the cell suspension slowly and with constant mixing. The mixture was incubated at 37 C for 10 minutes and then cooled in an ice bath. The EA suspensions prepared in this manner were stored at 4 C and used within 24 hours of preparation.

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* Plescia, O.J.; Amiraian, K.; Heidelberger, M. 1956. A kinetic method for the titration of complement. Arch. Biochem. Biophys. 62:346-354.

** Pennell, R.B. 1953. Blood cells and plasma proteins, p. 222. Academic Press, Inc., New York.

*** Mayer, M.M.; Croft, G.C.; Gray, M.M. 1948. Kinetic studies of immune hemolysis: I. A method. J. Exp. Med. 88:427-444.

B. C' SOURCES

Desiccated and standardized guinea pig C' (Difco) was stored at -40 C and was reconstituted just prior to use. As a source of human C', sera from two normal individuals were obtained and stored at -40 C until used.

C. KINETIC ASSAY

To 0.10 ml of EA suspension, 0.4 ml of diluted C' was added. The EA and C' were mixed thoroughly and transferred to a cuvette with a 1-mm path length. Absorbency readings were recorded at 700 mμ at 15- or 30-second intervals (depending on the speed of reaction) in a Beckman DK-2A spectrophotometer. Virtually all of the absorbency at this wavelength is caused by intact cells, and the effect of hemoglobin concentration is almost negligible. Readings were continued until the absorbency of the mixture had decreased to less than 0.05. This usually took less than 7 minutes.

III. RESULTS

The decrease in turbidity of the EA suspension in the presence of C' followed a first-order kinetics reaction after a short lag period (Fig. 1). The reaction can be described by the equation

$$K^1 = \frac{2.3}{t} \log \frac{A_0}{A_t} \qquad (1)$$

where K^1 is the first-order rate constant; t is the time (minutes) after addition of EA; A_0 is the absorbency immediately after addition of EA; and A_t is the absorbency at time t.

A series of curves is obtained (Fig. 2) when the change in turbidity for various known guinea pig C' concentrations is plotted as a first-order kinetics reaction [$\log (A_0/A_t)$ vs. t]. The slopes (k) of these curves in the linear range are equal to $K^1/2.3$ and are described by the equation

$$k = \frac{K^1}{2.3} = \frac{\log \frac{A_0}{A_2} - \log \frac{A_0}{A_1}}{t_2 - t_1} \qquad (2)$$

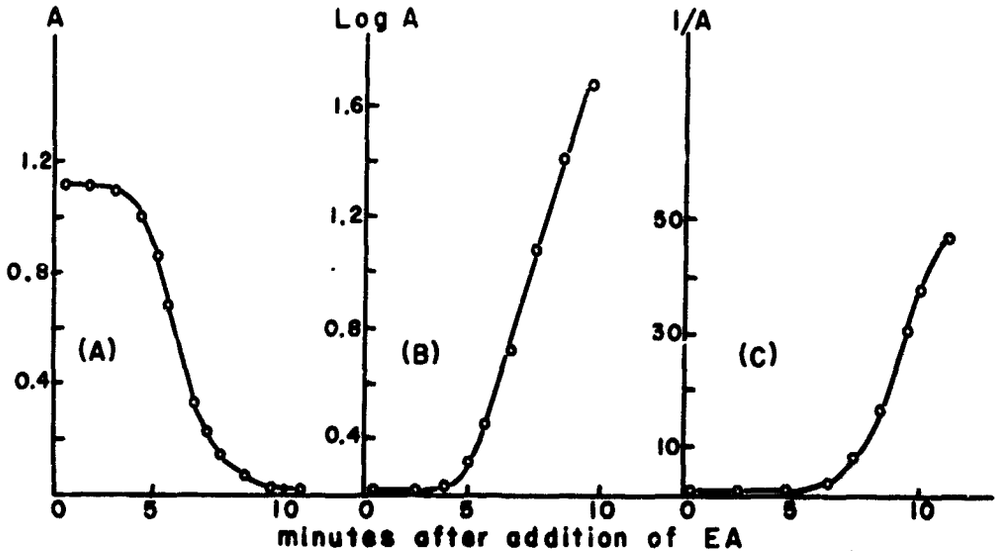

Figure 1. Decrease in Turbidity of EA Suspension in the Presence of C'. (A) Zero-order kinetics reaction; (B) first-order kinetics reaction; (C) second-order kinetics reaction.

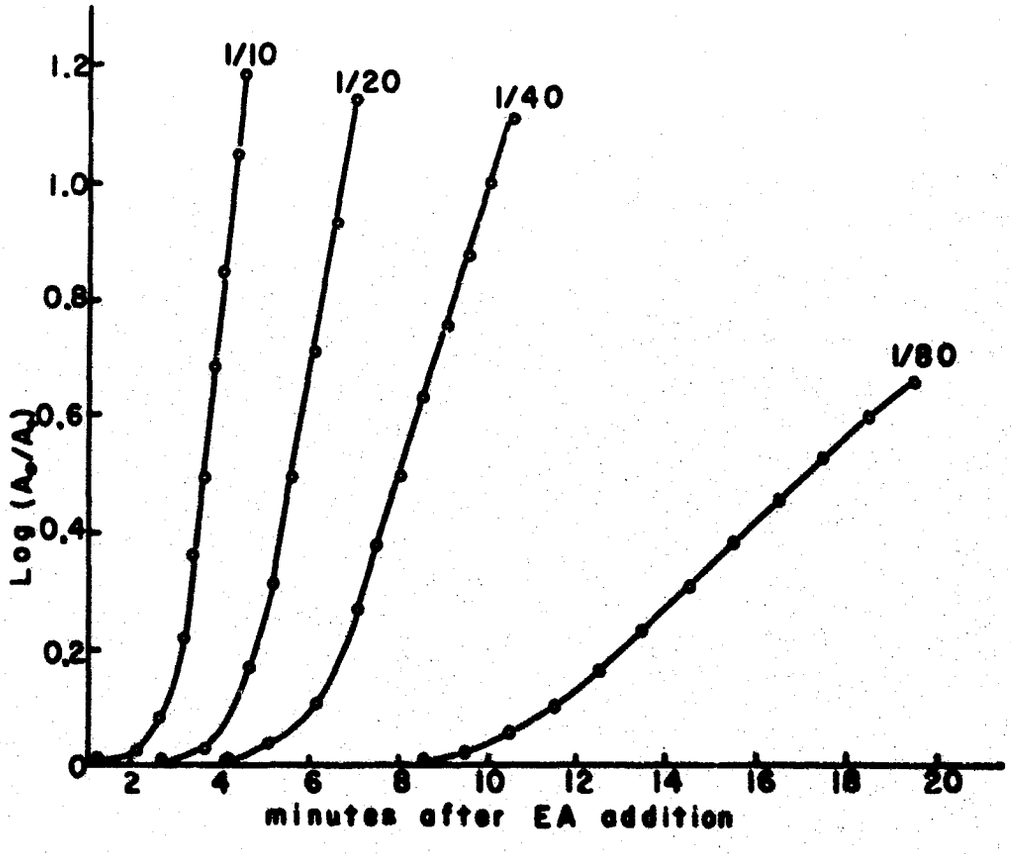

Figure 2. First-Order Kinetic Plots of Absorbency Decreases of EA Suspension in the Presence of Known Dilutions of Guinea Pig C'.

Equation (2) can be reduced to

$$k = \frac{\log A_1 - \log A_2}{t_2 - t_1} \qquad (3)$$

A linear standard curve is obtained when k for each C' concentration is plotted against C' concentration (expressed as the Naperian logarithm) as shown in Figure 3.

IV. DISCUSSION

An erythrocyte suspension containing 5×10^8 cells/ml proved to be the optimal concentration for C' determination. Although the range is increased when lower cell concentrations are used, the sensitivity is decreased, and the results are not as reproducible. This concentration of cells is in excess of that needed to saturate the system, and, as a result, an error in cell concentration as high as 10% has virtually no effect on the k value obtained. It is necessary to employ cuvettes with a short path length (e.g., 1 mm) because of the relatively high initial absorbency of the EA-C' mixture. Beckman DU and DK-2A spectrophotometers were employed in this laboratory, but any spectrophotometer adaptable to using a short-path cuvette would be suitable.

The concentration of hemolysin (1:80) used for erythrocyte sensitization gave the greatest change in slope with change in C' concentration. Hemolysin dilutions ranging from 1:10 to 1:320 were used, and the resulting EA suspensions were tested with known C' concentrations. When the slopes of the standard curves were compared (Fig. 4), a 1:80 dilution of hemolysin yielded the highest value. This dilution would then be expected to give the greatest sensitivity under these conditions.

It is possible to accurately determine the C' level of different animal and human sera by calculating the slope of the first-order plot. The slope can then be compared with reference curves prepared from standardized C' derived from sera of the same species.

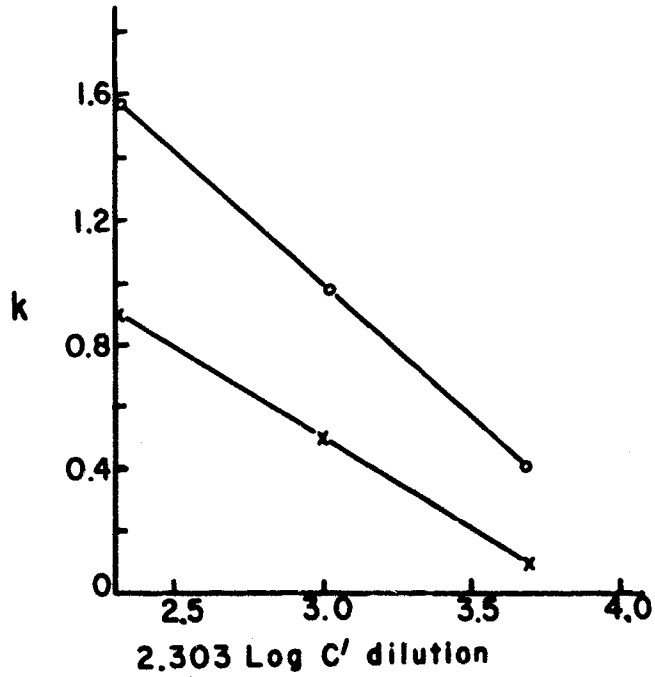

Figure 3. Kinetic Standard Curves for Guinea Pig C' (o) and Human C' (x). The k values are the slopes of the first-order kinetic plots for the C' dilutions.

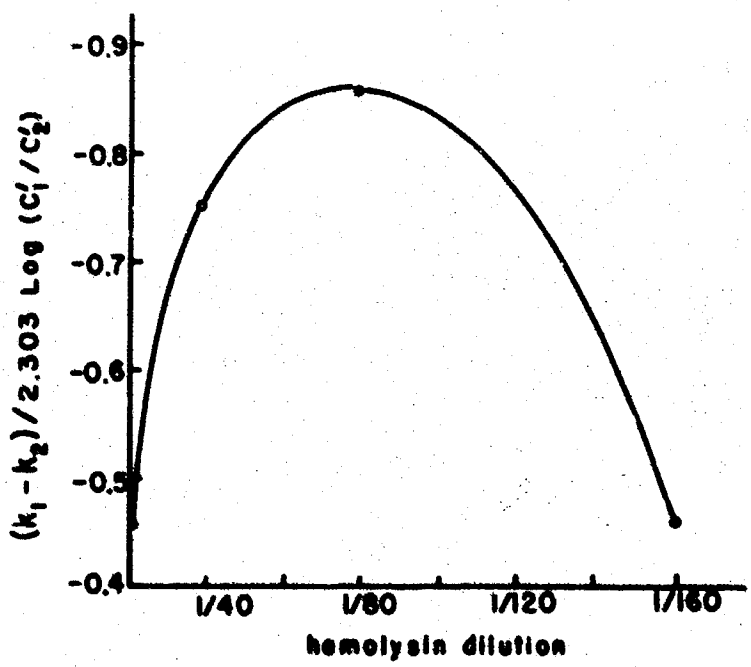

Figure 4. Effect of Hemolysin Concentration used for Erthrocyte Sensitization on the Slopes of the Standard Curves Obtained.

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14. Key Words

*Complement fixing
Hemolysis
Erythrocytes
*Kinetics
Lysis
*Spectrophotometry

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