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A Kinetic Flow Technic for Study of Immune Hemolysis.*† (24575)

LOUIS G. HOFFMANN,‡ HERBERT J. RAPP, JOSE R. VIÑAS§ AND MANFRED M. MAYER
Dept. of Microbiology, Johns Hopkins University, School of Medicine and School of Hygiene and Public Health, Baltimore, Md.

In previous publications from this laboratory(1,2), the lysis of sensitized sheep erythrocytes (EA; where E = erythrocyte, A = antibody to the Forssman hapten) by guinea pig complement (C′) has been shown to proceed according to the following mechanism:

$$EA \xrightarrow[Ca^{++}]{C'_1,C'_4} EAC'_{1,4} \xrightarrow[Mg^{++}]{C'_2} EAC'_{1,4,2} \xrightarrow{C'_a,C'_b} E^*$$

$EAC'_{1,4,2} \rightarrow$ Inactive product

$E^* \rightarrow$ Ghosts + hemoglobin

In this scheme, C'_1, C'_2 and C'_4 represent first, second and fourth components of C′ as defined by the conventional component reagents (3), while C'_a and C'_b are 2 components of C′ whose existence has recently been shown by Rapp(2); both are distinct from C'_1, C'_2 and C'_4, and either one or both of them are identical with the third component of C′, *i.e.*, C'_3, as defined in the conventional manner(3). C'_a and C'_b both act in absence of divalent cations. The symbols EA, $EAC'_{1,4}$ and $EAC'_{1,4,2}$ do not denote chemical compounds but intermediate products, *i.e.*, sensitized cells, sensitized cells which have reacted with C'_1 and C'_4, and sensitized cells which have reacted with C'_1, C'_4 and C'_2, respectively. E* represents a distinct intermediate which is capable of lysis without further interaction with C′ components. The present publication describes a technic by which formation and disappearance of each of these intermediates

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‡ Predoctoral Research Fellow, Am. Fn. for Allergic Dis., 1956-57.

§ Present address: Hemobanca, Barcelona, Spain.

may be visualized in a single experiment. The method represents an extension and refinement of technics employed in our earlier studies of appearance and disappearance of single intermediates(1).

Materials and methods. Preparation of veronal buffer, sheep erythrocyte (E) suspensions, EA, and of a reagent (C'_y) furnishing C'_2, C'_a and C'_b, as well as general experimental procedures for performing kinetic analyses of immune hemolysis, have been described(1). These were followed with minor modifications to be described in greater detail elsewhere. *Procedures.* A flask containing 6 x 10^9 EA and 1 ml of a 1/14 dilution of C′ in final volume of 90 ml with Ca^{++} (1.5 x 10^{-4} *M*) and Mg^{++} (5 x 10^{-4} *M*) present, was rocked continuously in water bath at 37.0°C. Aliquot samples of 1.5 ml were removed at desired intervals and mixed with 3 ml of ice-cold veronal buffer. They were centrifuged in the cold for 2-5 min. at 1000 g within 1 minute after withdrawal. Supernatant fluids were removed immediately after centrifugation and set aside for hemoglobin analysis, the tubes were drained and the lips wiped with absorbent paper. The sedimented cells were resuspended smoothly in *one* of the following reagents: 1 ml C'_y diluted 1/24 with respect to original serum in veronal buffer containing 10^{-3} *M* Mg^{++} (C'_y-Mg^{++}), 1.0 ml C'_y diluted 1/24 with respect to original serum in veronal buffer containing 5 x 10^{-3} *M* ethylene diamine tetraacetate (C'_y-EDTA), or 4.5 ml of plain veronal buffer. To retard further reactions of C′ in the samples after withdrawal and during these manipulations, the entire experiment was performed in cold room at 3-7°C. The resuspended cells were further incubated for 1 hour at 37°C. Then 3.5 ml of plain veronal buffer were added to those tubes which had previously received C'_y. All tubes were then centrifuged and the supernatant fluids were diluted 1/3 for hemoglobin determination at 410.5 mμ in a Beckman Model DU spectrophotometer. The supernatant fluids obtained after first centrifugation of samples were treated similarly.

Results. The data from a typical experiment (#100256) are shown in Fig. 1. The ordinates give the proportion of *original* cell

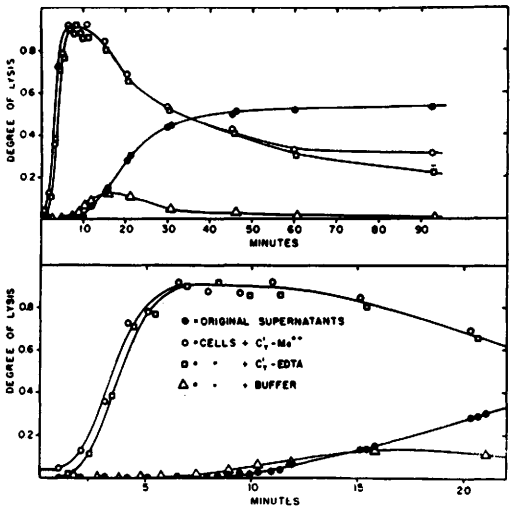

FIG. 1. Formation and disappearance of intermediates in immune hemolysis (Exp. #100256). Top portion: entire reaction. Bottom portion: early part of reaction on magnified time scale. Full explanation appears in text. Symbols have same meaning in both parts of figure. Reagent blanks for C'_y were obtained on samples drawn from flasks containing EA without C′, and subjected to the same treatment as samples from the flask containing EA and C′; avg degrees of lysis (4 determinations) were: .039 with C'_y-Mg^{++}, and .006 with C'_y-EDTA (both corrected for spontaneous lysis in buffer).

population which either had lysed (curve labeled "Original Supernatants") or could be lysed by the reagent indicated (other curves), at time of sampling indicated on abscissae.

Discussion. The differences among the curves obtained by exposing aliquots of cells from the same reaction mixture to different reagents reflect availability or unavailability of the various C′ components in these reagents; thus, plain veronal buffer allows only cells in the state E* to lyse; C'_y-EDTA furnishes C'_a and C'_b, as well as allowing lysis of E*; C'_y-Mg^{++} provides C'_2 in addition to C'_a and C'_b, and also permits lysis of E*. However, it would be erroneous to conclude that these cells lysed by C'_y-Mg^{++}, but not by C'_y-EDTA, are cells "in the state $EAC'_{1,4}$," in the terminology of our previous publications(1). Similarly, the proportion of cells lysed by C'_y-EDTA, after correction for spontaneous lysis of E*, cannot be equated to the proportion of cells "in the state $EAC'_{1,4,2}$." This criticism would also apply to Leon's

study of intermediates formed by human C′ (4).

To interpret data of this type correctly, it is necessary to use the "site concept" developed by Rapp(5) and described by Mayer (6). The concept distinguishes between molecular reactions taking place at individual sites on erythrocyte surface and the cellular event of hemolysis which is the consequence of these reactions. Thus, a sensitized site, SA, reacts first with C'_1 and C'_4 to form $SAC'_{1,4}$, and then with C'_2 to yield $SAC'_{1,4,2}$; the latter site either decays or reacts with C'_a and C'_b to become S*. A cell with at least 1 S* is in the state E*. Mathematical analysis of this model indicates that the proportion of cells lysed depends non-linearly on average number of $SAC'_{1,4,2}$ per cell and on concentration of C'_a and C'_b to which the sample of cells is exposed. Thus, the proportion of cells found to be "in the state $EAC'_{1,4,2}$" in the present system would vary with concentration of C'_a and C'_b in the reagent, making it impossible to interpret the results in these terms.

In terms of "site concept" then, the meaning of curves in Fig. 1 is as follows: curve labeled "Original Supernatants" shows progress of lysis; curve labeled "Cells + Buffer" shows proportion of E*; curve labeled "Cells + C'_y-EDTA" shows proportion of E* plus proportion of cells lysed as a result of the presence of $SAC'_{1,4,2}$, the difference between this curve and the "Cells + Buffer" curve being a measure of the average number of $SAC'_{1,4,2}$ per cell; curve labeled "Cells + C'_y-Mg^{++}" shows proportion of E* plus proportion of cells lysed as a result of the presence of $SAC'_{1,4,2}$ plus proportion of cells lysed as a result of the presence of $SAC'_{1,4}$ on the same cells, which are converted to $SAC'_{1,4,2}$ by the C'_2 furnished by the reagent. Thus, the difference between the "Cells + C'_y-Mg^{++}" and "Cells + C'_y-EDTA" curves is a measure of the average number of $SAC'_{1,4}$ per cell, except where the 2 curves coincide as a result of the presence of a sufficient number of $SAC'_{1,4,2}$ per cell to yield virtually complete hemolysis with concentration of C'_a and C'_b added, in which event formation of further $SAC'_{1,4,2}$ from $SAC'_{1,4}$ by reaction with the C'_2 added with the reagent makes no difference.

The technic here described furnishes a means whereby C′ from different species may be compared(7) and the effect of C′ inhibitors studied(8) without recourse to use of conventional reagents for the C′ components(3), to which serious objections have been stated by Mayer(6). These and other applications are presently under study in this laboratory.

Summary. A method for the kinetic analysis of the formation and disappearance of intermediates in immune hemolysis has been described. The interpretation of results obtained with it has been discussed, and certain applications indicated.

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