The Third Component of the Guinea Pig Complement System. II. Kinetic Study of the Reaction of EAC'4,2a with Guinea Pig C'3. Enzymatic Nature of C'3 Consumption, Multiphasic Character of Fixation, and Hemolytic Titration of C'3*

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ABSTRACT: Kinetic analyses of C'3 consumption and SAC'4,2a,3 formation indicate that fixation of C'3 is a multiphasic process. The initial velocity of C'3 consumption varies with the concentrations of EAC'4,2a and C'3 according to Michaelis–Menten kinetics. The cell-bound C'3 is stable. For the subsequent reaction with C'5 cell-bound C'2a and C'3 are required. A procedure for hemolytic titration of C'3 is presented.

The concepts and experimental tools used in the present work on C'3[1] are essentially similar to those employed in preceding studies of C'1, C'4, and C'2. Therefore, it will be helpful to review briefly the early reaction stages of the C' system.

In the first step of the hemolytic process mediated by antibody and C', antigen–antibody complexes are formed on the erythrocyte membrane by addition of appropriate antibody to erythrocytes. These antigen–antibody complexes adsorb C'1 and change it to an active enzyme (Lepow *et al.*, 1954) designated C'1a, which then mediates the fixation of C'4, mostly to the cell membrane (Müller-Eberhard and Lepow, 1965). This process is rather inefficient and only a small proportion of the C'4 becomes fixed while the greater part ends up as an inactive derivative in the fluid phase of the reaction system.

The next reaction step, also mediated by C'1a, involves the fixation of C'2 to receptors on the previously fixed C'4 molecule (Stroud *et al.*, 1965; Sitomer *et al.*, 1966). Detailed studies of this process have shown that C'1a cleaves the C'2 molecule into a fragment, C'2a, which becomes bound to C'4 and a fragment, C'2i, which is hemolytically inactive and which ends up in the fluid phase of the reaction system (Mayer, 1965). The hypothesis has been advanced that C'1a opens a susceptible bond in the C'2 molecule, thus exposing a reactive group on the C'2a fragment which then binds to a receptor on C'4, provided a C'4 molecule is immediately available for reaction (Stroud *et al.*, 1966). If this is not the case, the C'2a fragment loses its capacity to combine with C'4 and it is thought that this may occur by combination of the reactive group on C'2a with a molecule of water instead of C'4. This interpretation is derived from the fact that incubation of C'2 with C'1a in the fluid phase, *i.e.*, in the absence of cells, antibody, and C'4, results in the formation of fragments of C'2 which are hemolytically inactive (an essentially similar concept has been proposed for the fixation of C'4 by C'1a, except that no fragments of C'4 have been found (Müller-Eberhard and Lepow, 1965)).

The cell-bound C'4,2a complex then acts on C'3 and mediates its fixation, probably to receptors on the cell membrane. In a study of this reaction with ^{125}I-labeled C'4 and ^{131}I-labeled C'3, Müller-Eberhard *et al.* (1966) observed that one C'4,2a complex mediated the fixation of several hundred molecules of radioiodinated C'3. On this basis he proposed that C'3 becomes "activated" by the C'4,2a complex, the latter acting like an enzyme. Some of the activated C'3 becomes fixed to the cell membrane, while the remainder ends up in the fluid phase in inactive form. This hypothesis of C'3 fixation is also essentially similar to that proposed for the fixation of C'2. Indeed, recent experiments indicate that the C'3 molecule, like C'2, is broken into several fragments and, presumably it is one of these fragments that becomes fixed to the cell (Mayer *et al.*, 1967).

When highly purified C'3 became available, as described in the preceding paper (Shin and Mayer, 1968a), we started a quantitative and kinetic study of its reaction with EAC'4,2a by hemolytic methods along two lines of approach, namely, kinetic and quantitative studies of the disappearance or consumption of hemolytic C'3 activity and kinetic studies of the formation of sites in the state SAC'4,2a,3. The results of these experiments are presented in this paper.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: E, sheep erythrocyte; A, antibody to the boiled stromata of sheep erythrocytes; S, site on the E surface capable of reacting with A and complement components; C', complement; C'*j*, *j*th component of complement; C'*j*a, activated form of C'*j*; SAC'*i,j,k*, S which has reacted with A and C'*i*, C'*j*, and C'*k*; EAC'*i,j,k*, E carrying at least one SAC'*i,j,k* plus any number of precursor sites of SAC'*i,j,k* in the hemolytic sequence but none of its successors; S*, S which has reacted with A and all complement components.

Materials and Methods

Complement Components. Purified guinea pig C'3 (lot 9, 43 μg of nitrogen/ml by micro-Kjeldahl analysis and 10,000 units of C'3/ml), prepared as described (Shin and Mayer, 1968b), was used in all of the reported experiments. All experiments were repeated with another batch of C'3 (lot 7, 22 μg of nitrogen and 5000 units of C'3 per ml), prepared in the same manner. Partially purified C'2 was made by the method of Borsos *et al.* (1961a). Methods described by Nelson *et al.* (1966) were used for the preparation of partially purified C'5, C'6, C'7, C'8, and C'9, except that C'9 was further purified by disc electrophoresis in polyacrylamide gel (Davis, 1964) in order to remove contaminating C'3. Briefly, guinea pig pseudoglobulin separated at pH 7.5 and ionic strength 0.04 was purified on DEAE- and CM-cellulose columns, as described by Nelson *et al.* (1966). The C'9 pool was concentrated by ultrafiltration. Approximately 200–300 μg of protein containing C'9 was mixed with an equal volume of 10% sucrose and layered on the large pore polyacrylamide gel. The inside diameter of the glass tube containing the gel was 1.4 cm and the large pore and small pore gels were 1 and 7 cm long, respectively. The small pore gel contained 7.0% acrylamide and 0.2% bisacrylamide. Electrophoresis was run at 3–6°, 5 mA at 150 V, until the band of contaminating hemoglobin emerged from the anodal end of the small pore gel. Under these conditions of electrophoresis, C'9 was the fastest and C'3 the slowest moving complement component. The small pore gel was sliced into 3.5-mm segments, each of which was eluted three times in 3 ml of buffer, for 6 hr. The recovery of C'9 from the gel was about 60%.

The titers of complement components fluctuated widely in analyses performed on different days due to unknown factors. Therefore, a standard lot of each complement component was arbitrarily chosen and assigned a titer, expressed in units per milliliter. Each new batch was then calibrated by reference to the standard lot. The arbitrarily assigned titer of the standard lot was chosen so as to be roughly the same as the dilution required for lysis of 50% of the cells in the titration procedure described by Nelson *et al.* (1966). Thus, titers of 1000 units/ml were assigned to the standard lots of C'5, C'6, and C'7 and titers of 10,000 units/ml were assigned to the standard lots of C'8 and C'9.

Buffers. Buffer A was composed of isotonic Veronal–NaCl buffer containing 0.1% gelatin (pH 7.3), ionic strength 0.147, prepared as described in (Kabat and Mayer, 1961). Buffer B contained nine parts of buffer A and one part of 0.1 M EDTA. The pH of 0.1 M EDTA was adjusted to 7.3 with saturated aqueous NaOH solution. Buffer C contained one part of buffer A and one part of 5% aqueous glucose solution containing 0.1% gelatin. When necessary Ca^{2+} and Mg^{2+} were added to buffer A or C to a final concentration of 0.15 and 1.0 mM, respectively. The so modified solutions are designated buffer A^{2+} and buffer C^{2+}. All experiments were performed in buffer C^{2+} unless stated otherwise. In studies of the effect of ionic strength, NaCl in buffer A was replaced with the appropriate amount of glucose, mannitol, or sucrose, so as to maintain isoosmolarity. In studies of the effect of pH, buffer C was made with varying amounts of HCl.

Erythrocytes Carrying Antibody and Complement Components. EAC'1a,4 were prepared as described previously (Mayer and Miller, 1965). Only batches of cells without immune adherence reactivity were used. T_{max} of such cells varied between 6 and 20 min. EAC'4 were prepared as described by Borsos and Rapp (1963). EAC'1a,4,2a were made by incubating 50 ml of EAC'1a,4 (1.54×10^8 cells per ml) with an equal volume of C'2 (32–64 units/ml) for the T_{max} time at 30°. At the end of the incubation period, 100 ml of ice-cold buffer C^{2+} was added to slow down the reaction, the cells were sedimented by centrifugation, washed once, and standardized to the desired cell concentration in an appropriate buffer.

EAC'4,2a were made from EAC'1a,4,2a by washing twice with ice-cold buffer B followed by incubation in buffer B at 37° for 5 min. They were then washed twice in ice-cold buffer A^{2+} and suspended in an appropriate buffer. The cells had about eight to ten SAC'4,2a per cell by the method of Borsos *et al.* (1961b). EAC'1a,4,3 were prepared by incubating EAC'1a,4 (1.54×10^8 cells per ml) at 30° for 60 min with an equal volume of a mixture containing 0.2 unit of C'2 and 50 units of C'3 per ml. The cells were chilled by dilution with ice-cold buffer C^{2+}, sedimented by centrifugation in the cold, washed twice, incubated for 1 hr at 37° for decay of C'2, washed again, and suspended to 1.54×10^8 cells per ml.

Converting Reagents.[2] In studies of the reaction of C'3 with SAC'42a the resulting SAC'4,2a,3 were converted into S* with a converting reagent containing C'5 (50 units), C'6 (25 units), C'7 (25 units), and C'8 (50 units) and C'9 (125 units) per milliliter in buffer C. This is designated as converting reagent A.

In titrations of C'3, as described in the next paragraph, the following converting reagent was used: C'2 (80 units), C'5 (100 units), C'6 (100 units), C'7 (300 units), C'8 (200 units), and C'9 (300 units) per milliliter in buffer C^{2+}. This is designated as converting reagent B.

Titration of C'3. During the initial phases of this study, C'3 was titrated with EAC'4,2a and converting reagent A in a manner patterned after the C'2 titration of Borsos *et al.* (1961a,b). However, this approach suffers from several practical difficulties and, therefore, a simplified procedure was designed in which two parts of C'3 sample to be titrated were mixed at 0° with two parts of converting reagent B. One part of ice-cold EAC'1a,4 (1.54×10^8 cells per ml) was then added and all the tubes were transferred to the 37° water bath for 90-min incubation with frequent shaking. At the end of this period, ten parts of 0.15 M NaCl solution were added and the tubes were centrifuged. The degree of lysis was determined by analysis of the supernatant fluids for oxyhemoglobin at 412 mμ with the Beckman DU spectrophotometer, and converted into the average number of

[2] In this instance this term refers to conversion of SAC'4,2a,3 into S*. This should not be confused with the term conversion as used by Müller-Eberhard *et al.* (1967) to describe the inactivation of C'3 by C'4,2a.

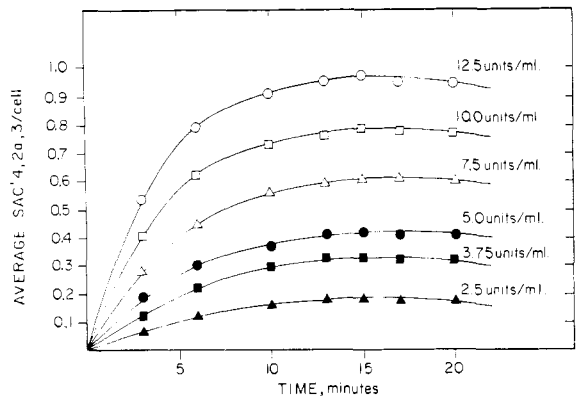

FIGURE 1: Kinetics of SAC'4,2a,3 generation at different C'3 concentrations, as indicated.[3]

SAC'4,2a,3 per cell.[3] Hemolysis due to contaminating C'3 in the converting reagent was less than 3%.

Kinetic Studies of Reaction between EAC'4,2a and C'3. All experiments were done at 25° unless noted otherwise. At this temperature the half-life of EAC'4,2a is about 70 min. Therefore, kinetic measurements during the first few minutes of reaction would not be significantly affected by the decay of EAC'4,2a. In order to raise the temperature of the EAC'4,2a suspension quickly from 0 to 25°, a suspension containing 1.54×10^9 cells per ml (unless indicated otherwise) was diluted tenfold in buffer C^{2-}, previously warmed to 20°, followed by continuous shaking for 1 min. At 1.5 min an equal volume of C'3, also at 25°, was added to start the reaction. At appropriate times, 0.50-ml samples were taken, immediately diluted tenfold in ice-cold buffer C^{2+} and centrifuged for 5 min at 2000*g*. The supernatant fluids were then poured off and saved for titration of the C'3 remaining in the fluid phase of the reaction system. The cells were washed once in buffer C and suspended to 1.54×10^8 cells per ml in buffer C. One part of the cell suspension was incubated with four parts of converting reagent A for 90 min at 37°. The degree of lysis was measured by hemoglobin analysis as described in the C'3 titration procedure. In some experiments, kinetic measurements were restricted to analysis of C'3 remaining in the fluid phase. For this purpose, 1.5-ml samples were quickly filtered at room temperature (25–26°) through a Pasteur pipet stuffed with a Millipore prefilter, as described by D. J. Hingson, K. A. Massengill, and M. M. Mayer (unpublished work). Loss of C'3 on the filter was avoided by incorporation of 0.25% gelatin in the reaction mixture.

Results

Kinetics of Generation of SAC'4,2a,3 as a Function of C'3 Concentration. Kinetic analyses were performed with reaction mixtures containing 2.5, 3.75, 5.0, 7.5, 10.0, and 12.5 units of C'3 per ml, respectively. The results, plotted in Figure 1, show that maximal lysis was reached in 15 min, regardless of the C'3 concentration. A plot of the maxima *vs.* C'3 concentration yielded a slightly curved line, concave to the abscissa.

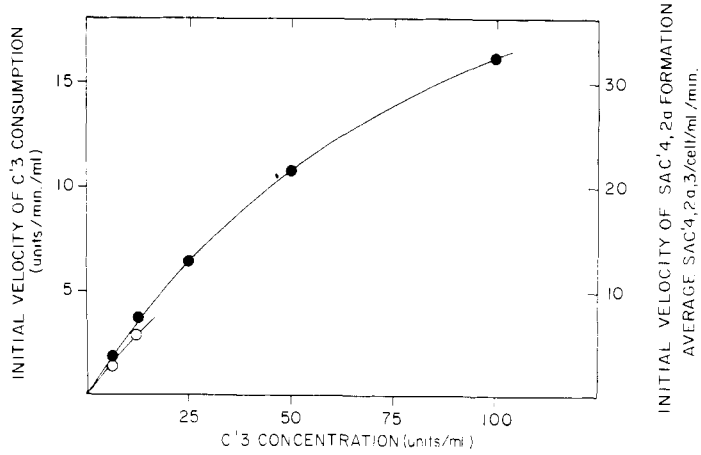

FIGURE 2: Initial velocity of C'3 consumption and SAC'4,2a,3 formation as a function of C'3 concentration. (●—●) C'3 consumption; (○—○) SAC'4,2a,3 generation.

Initial Velocities of C'3 Consumption and SAC'4,2a,3 Generation as a Function of C'3 Concentration. Kinetic analyses were performed with reaction mixtures containing 6.25, 12.5, 25, 50, and 100 units of C'3 per ml. Four successive samples were taken during the first 4 min of reaction and each of these was titrated for C'3 in the fluid phase and SAC'4,2a,3 on the cells. The method of Nelson and Dawson (1944) was used for calculating initial reaction velocities. A plot of the initial velocity of C'3 consumption *vs.* concentration of C'3 yielded a curve concave to the abscissa, as shown in Figure 2. This indicates that the consumption velocity approaches a limit at very high concentration of C'3. A double-reciprocal plot of the data gave a straight line from which a V_{max} value of 36 units of C'3 min^{-1} ml^{-1} was estimated. The data on initial velocity of SAC'4,2a,3

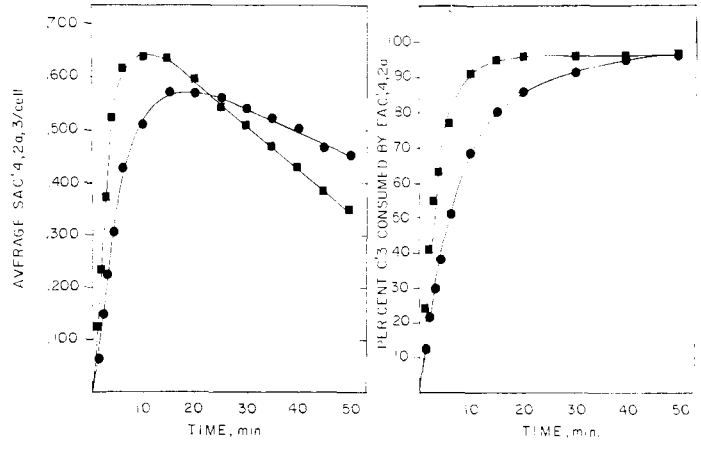

FIGURE 3: Kinetics of SAC'4,2a,3 generation (left) and C'3 consumption (right) as a function of EAC'4,2a concentration. (■—■) EAC'4,2a concentration of 7.7×10^7 per ml; (●—●) EAC'4,2a concentration of 3.85×10^7 cells per ml.

[3] The average number of SAC'4,2a,3 per erythrocyte is given in terms of $-\ln(1 - y)$, where y represents degree of lysis, expressed as a fraction of 1 (Kabat and Mayer, 1961).

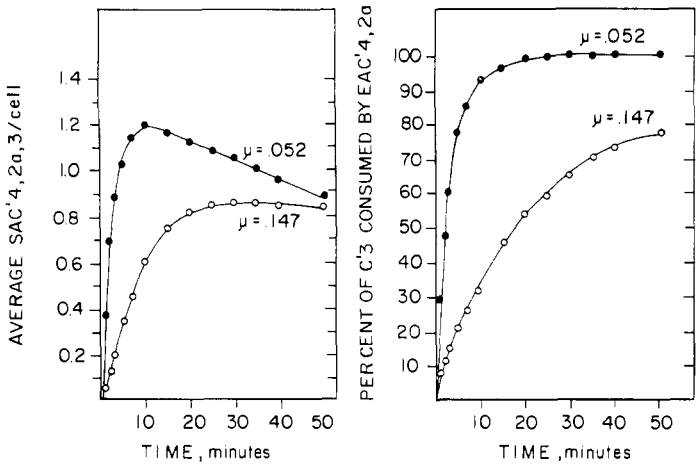

FIGURE 4: Kinetics of SAC'4,2a,3 formation (left) and C'3 consumption (right) at different ionic strengths, as indicated.

formation, also shown in Figure 2, are of limited value as analyses were restricted to concentrations of C'3 giving less than 70% lysis.

Kinetics of Formation of SAC'4,2a,3 and Consumption of C'3 as a Function of SAC'4,2a Concentrations. Two EAC'4,2a suspensions, adjusted to concentrations of 1.54×10^8 and 7.7×10^7 cells per ml, respectively, were mixed and incubated with equal volumes of C'3 containing 20 units/ml, and the resulting reaction mixtures were designated A and B, respectively. The usual 0.5-ml samples were taken from reaction mixture A and diluted with 4.5-ml portions of ice-cold buffer C^{2+}, while samples of 1.0 ml were taken from reaction mixtures B and diluted with 9.0-ml portions of ice-cold buffer C^{2+}. Subsequent treatments of the samples were the same. The results, shown in Figure 3, indicate that the initial velocities of SAC'4,2a,3 formation and C'3 consumption in A were twice as great as those in B.

The SAC'4,2a formation curves in Figure 3 rise to a maximum and then decline due to decay release of C'2a (as shown in a later section, cell-bound C'3 does not decay). There is a shift in T_{max} resembling that observed in the reaction between SAC'1a,4 and C'2 (Borsos *et al.*, 1961b) and there is a corresponding differential between the maximal levels of SAC'4a,2,3, presumably due to the different periods of decay.

A similar experiment covering the cell concentrations

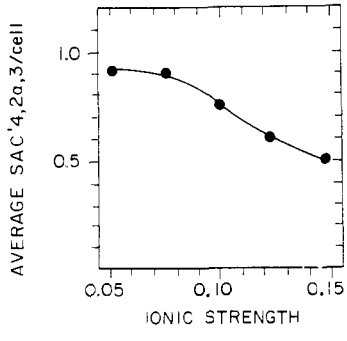

FIGURE 5: Formation of SAC'4,2a,3 at different ionic strengths, as indicated.

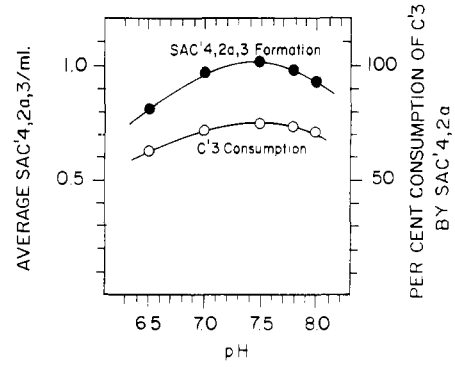

FIGURE 6: Effect of pH variation on SAC'4,2a,3 formation and C'3 consumption, as indicated.

ranging between 2 and 8×10^8 cells per ml was performed. As shown in the previous experiment, direct proportionality between initial velocity of C'3 consumption and concentration of EAC'4,2a was observed.

Effect of Ionic Strength. A kinetic experiment comparing ionic strengths of 0.147 and 0.052 is shown in Figure 4. It is evident that the initial velocities of both C'3 consumption and SAC'4,2a,3 formation are about six times greater at the lower ionic strength. On the other hand, maximal site formation and C'3 consumption did not differ greatly. Indeed, most, if not all of the difference may be attributed to decay release of C'2a. The site formation curves show a difference in T_{max} which indicates that increase in ionic strength simulates a decrease in the concentration of SAC'4,2a (*cf.* Figure 3).

A more extensive experiment on the effect of ionic strength, covering the range from 0.052 to 0.147, is shown in Figure 5. Isoosmolarity was maintained by replacing NaCl with glucose. The reaction time was 10 min.

Evidence was also obtained that the extent of reaction at any given ionic strength is influenced by the choice of sugar for balancing osmolarity. Thus, small differences were noted at ionic strength 0.07 between glucose, mannitol, and sucrose.

Effect of pH. Measurements with a reaction period of 10 min at ionic strength 0.15 indicated a pH optimum of 7.5 for SAC'4,2a,3 formation and C'3 consumption, as shown in Figure 6.

Effect of Temperature. It was found that the initial velocities of SAC'4,2a,3 formation and C'3 consumption increased with temperature between 15 and 25°, the $Q10$ being 2.5. The same temperature coefficient was noted for C'3 consumption between 0 and 15°, but a much larger temperature coefficient was observed for the formation of SAC'4,2a,3 in the low-temperature range, the $Q10$ being about 10 between 0 and 10°. Accordingly, as shown in Figure 7, an Arrhenius plot yielded a straight line for the consumption of C'3, while formation of SAC'4,2a,3 followed a diphasic course.

Stability of Fixed C'3. It was shown by Inoue and Nelson (1966) that C'2a is lost from EAC'1a,4,2a,3 by decay release at the same rate as from EAC'4,2a. The hemolytic activity of such decayed cells was only partially restored by C'2 (Nishioka and Linscott, 1963).

This question was reexamined more rigorously with

EAC'1a,4,3 prepared as described in the section on Materials and Methods. One portion of the cells was incubated at 25° and another at 37°. Samples were taken at suitable intervals of time and treated with converting reagent B. There was essentially no decrease of hemolytic activity with time, indicating that fixed C'3 is stable.

Role of Fixed C'2a and C'3 in Consumption of C'5. EAC'1a,4,2a and EAC'1a,4,2a,3 were prepared from EAC'1a,4 (1.54×10^8 cells per ml) by treatment for 10 min at 30° with an equal volume of buffer C^{2+} containing either 75 units of C'2 per ml or 75 units of C'2 and 100 units of C'3 per ml. The cell suspensions were then chilled, diluted, and centrifuged in the cold. The supernatant fluid was poured off and the sedimented cells were washed once, followed by resuspension to a concentration of 1.54×10^8 cells per ml. A portion of the EAC'1a,4,2a,3 preparation was allowed to decay for 90 min at 37°, washed once, and the resulting EAC'1a,-4,3 were standardized again to 1.54×10^8 cells per ml. Each of the three cell preparations (*i.e.*, EAC'1a,4,2a, EAC'1a,4,3, and EAC'1a,4,2a,3) was incubated with 10 units of C'5 for 6 min at 30°. There was no loss of C'5 in the reaction mixtures with EAC'1a,4,2a or EAC'1a,4,3, but in the reaction mixture with EAC'1a,4,2a,3 five units of C'5 were consumed.

Titration of C'3. During the early phases of this work attempts were made to titrate C'3 by a procedure involving two successive steps, *i.e.*, the reaction between EAC'4,2a, and C'3, and conversion of the resulting SAC'4,2a,3 into S* with converting reagent A. This was patterned essentially after the procedure of kinetic analysis. However, it emerged in practice that there are three serious disadvantages due to the instability of EAC'4,2a, *viz.*, these cells have to be prepared daily, they cannot be prewarmed to the reaction temperature without some loss of reactivity, and uncontrollable experimental fluctuations arise in the conversion of SAC'4,2a,3 into S* because of variations in loss of C'2a during washing of the reaction intermediate. In order to overcome these difficulties a titration procedure was devised in which EAC'1a,4 are incubated with appropriate dilutions of C'3 and conversion reagent B, as described in the section on Materials and Methods. A titration of C'3 performed in this manner is shown in Figure 8 as a plot of $-\ln(1 - y)$ *vs.* concentration of C'3. As shown in Figure 8, the response curve is usually entirely concave to the abscissa, but we have encountered a few analyses in which slightly sigmoidal response curves were obtained. We do not know the reason for this exceptional behavior except that it has been noted only with certain batches of EAC'1a,4.

Discussion

For the purpose of comparative evaluation, most of the kinetic experiments in the present study were performed by a dual approach, namely, measurement of C'3 consumption and analysis of SAC'4,2a,3 formation. Since assays of C'3 consumption are technically simpler and more flexible this technique yielded more experimental data covering a wider range of experimental variables than analyses of SAC'4,2a,3 generation. For this reason, the relationships between initial reaction velocity and the concentrations of EAC'4,2a and C'3 could be studied extensively only by the technique of C'3 consumption. As expected, the results of these studies, shown in Figures 1–5, conformed to Michaelis–Menten kinetics which supports the hypothesis that the C'4,2a complex acts like an enzyme (Müller-Eberhard *et al.*, 1967). V_{max} was about 3000 molecules of C'3 consumed/SAC'4,2a per min at 25°, provided that our C'3 preparation is chemically pure.

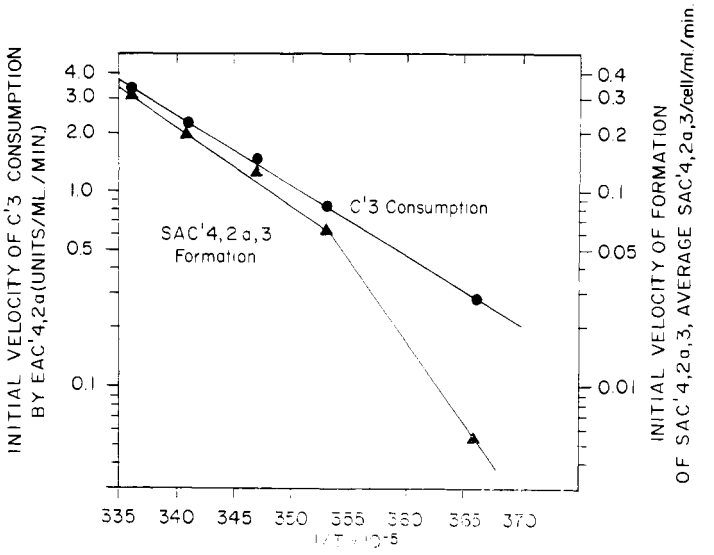

FIGURE 7: Arrhenius plots of the initial velocity of SAC'4,-2a,3 formation and C'3 consumption, as indicated.

One of the most interesting aspects of the present work emerged from measurements of the initial velocities of C'3 consumption and SAC'4,2a,3 formation at different temperatures. As shown in Figure 7, an Arrhenius plot of C'3 consumption gave a straight line over the entire range of temperatures studied, while that for SAC'4,2a,3 formation followed a diphasic course. Between 37 and 10° the Arrhenius plot for site formation ran parallel to that for C'3 consumption, but below 10° the velocity of site formation dropped much more rapidly.

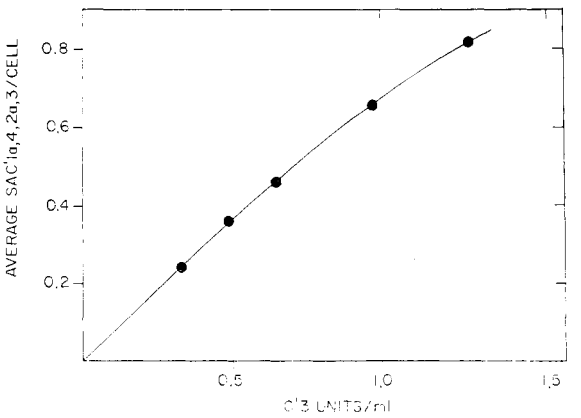

FIGURE 8: Dose response curve. Formation of SAC'1a,-4,2a,3 expressed as $-\ln(1 - y)$ *vs.* C'3 concentration.

In order to interpret this, let us recall Müller-Eberhard's proposal that C'3 becomes "activated" by the C'4,2a complex, the latter acting like an enzyme. The present finding that the destruction of C'3 by the C'4,2a complex follows Michaelis–Menten kinetics would be in agreement with this hypothesis. It is reasonable to assume, by analogy with the hypothesis for the reaction of C'2 with SAC'1a,4 that a susceptible bond is opened in the C'3 molecule by the postulated C'4,2a enzyme, exposing a reactive group which may then combine either with certain receptors on the cell membrane or with other compounds, possibly including water, the latter reaction producing an inactive derivative of C'3. This hypothesis would be in accord with recent evidence that the C'3 molecule breaks into several fragments on reaction with C'4,2a (Mayer *et al.*, 1967; Müller-Eberhard *et al.*, 1967). It follows that the fixation of C'3, or of a fragment of the C'3 molecule, is preceded by a fission process. The data in Figure 7 then indicate that at elevated temperatures this fission process constitutes the rate-controlling step in the formation of SAC'4,2a,3 sites, while at low temperature some other process, possibly the actual binding, controls the fixation rate. This means that the formation of SAC'4,2a,3 is a multiphase process and in support of this view is the finding, presented in the following article (Shin and Mayer, 1968b), that various aromatic compounds interfere with C'3 fixation even though they do not affect the destruction of C'3 by C'4,2a.

Efficiency of C'3 Fixation. If we assume that our C'3 preparation is chemically pure, it can be estimated that about 300 C'3 molecules are required in the reaction with EAC'4,2a in order to create one site in the state EAC'4,2a,3. There are probably at least three reasons for this great inefficiency. First, it is likely that some of the "activated" C'3 molecules, or C'3 fragments, fail to combine with receptors on the cell membrane and end up in the fluid phase in inactive form. Secondly, as shown by Müller-Eberhard *et al.* (1966) the number of C'3 molecules which becomes fixed to the cell membrane greatly exceeds the number of bound C'4 molecules and, hence, many C'3 molecules will become bound at loci remote from SAC'4,2a. Such C'3 molecules could not be expected to function in the subsequent reaction with C'5 and, therefore would be hemolytically inactive. Thirdly, we are not certain that the converting reagent is maximally efficient.

Acknowledgment

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