The Third Component of the Guinea Pig Complement System. III. Effect of Inhibitors*

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ABSTRACT: *N*-Acetyl-L-tyrosyl ethyl ester and certain other aromatic compounds inhibit the fixation of C'3 by EAC'4,2a.

N-Acetyl-L-tyrosyl ethyl ester also inhibits the subsequent reaction steps with C'5 and C'6. However, these inhibitors do not block the destruction of C'3 by EAC'4, 2a. Neither do they affect native C'3 in solution or cell-bound C'3.

Basch (1965) has shown that certain aromatic compounds inhibit the lysis of the intermediate EAC'1a, 4,2a by the late-acting complement components of guinea pig serum. While he could not make a definitive identification of the reaction step susceptible to inhibition, his results suggested that the reaction between EAC'4,-2a,3 and C'5 is inhibited. Müller-Eberhard *et al.* (1966) studied human C'3 and found that phloridzin inhibits its fixation on the cell membrane. The present work on guinea pig C'3, described in the preceding papers (Shin and Mayer, 1968a,b), made it possible to study this problem further.

Materials and Methods

Complement Components. These were obtained by the methods described in Shin and Mayer (1968a,b).

Buffer. Buffers were made as described in the preceding paper. Buffer C^{2+} was used in all experiments except in agglutination studies in which buffer B was used.

Erythrocytes Carrying Antibody and Complement Components. EAC'4,2a were made as described in Shin and Mayer (1968a,b). EAC'4, 2a,3, with a low number of SAC'4,2a,3 sites per cell, were made by reaction of one part of ice-cold EAC'4,2a (1.54×10^9 cells per ml) with 19 parts of prewarmed C'3 (2.5 units/ml) for 12 min at 30°. The reaction mixture was immediately diluted fivefold with ice-cold buffer and centrifuged. The cells were washed twice with ice-cold buffer and adjusted to the desired concentration.

EAC'1a,4,2a,3, with a high number of SAC'1a,4,2a,3 per cell, were made by reacting prewarmed EAC'1a,4 (1.54×10^8 cells per ml) with an equal volume of prewarmed buffer containing 80 units of C'2 and 140 units of C'3 per ml, for 12 min at 30°. The reaction mixture was promptly diluted with ice-cold buffer, centrifuged, and the cells were washed twice before adjustment to the desired concentration.

EAC'1a,4,2a,3,5 were prepared by reaction of prewarmed EAC'1a,4 (1.54×10^8 cells per ml) with an equal volume of prewarmed buffer containing 30 units of C'2 and 90 units of C'3 per ml, for 12 min at 30°. The cells were diluted with ice-cold buffer, centrifuged, and washed once. The resulting EAC'1a,4,2a,3 cells were mixed with seven parts of prewarmed C'5 (85 units/ml) for 20 min at 30°. Again the cells were chilled, diluted, centrifuged, and washed once, followed by standardization to the desired concentration.

Converting Reagents. The converting reagent for EAC'4,2a,3, designated as converting reagent A, was made as described in Shin and Mayer (1968b). The converting reagent for EAC'4,2a,3,5 contained C'6 (83 units/ml), C'7 (83 units/ml), C'8 (63 units/ml), and C'9 (210 units/ml) and the converting reagent for EAC'1a, 4,2a,3,5,6 contained C'7 (33 units/ml), C'8 (33 units/ml), and C'9 (83 units/ml).

Kinetics Analyses of Complement Component Reactions. The techniques described in the preceding paper (Shin and Mayer, 1968b) were used. Briefly, cellular intermediates, kept at 0°, were brought to the desired temperature by diluting tenfold in prewarmed buffer to a final concentration of 1.54×10^8 cells per ml and equilibrating for 1.5 min. They were mixed with an equal volume of the prewarmed complement component, either with or without inhibitor. At appropriate times, 0.5-ml samples were pipetted into 2.5 ml of ice-cold buffer and centrifuged. The cells were washed once and resuspended in 0.25 ml of buffer. They were then mixed with 1.0 ml of the appropriate converting reagent at 0°, followed by transfer to the 37° water bath for the 90-min incubation.

Inhibitors. Inhibitors were dissolved in buffer C^{2+} and pH was adjusted to 7.3 with 1 N HCl or NaOH, if necessary. Epinephrine was dissolved in a small drop of 1 N HCl, followed by buffer C^{2+} and adjustment of pH. Most inhibitors tested did not alter the conductivity of the buffer and the ones which did, caused only a minor increase. A control with extra NaCl was used to simulate the increment of ionic strength due to the inhibitor.

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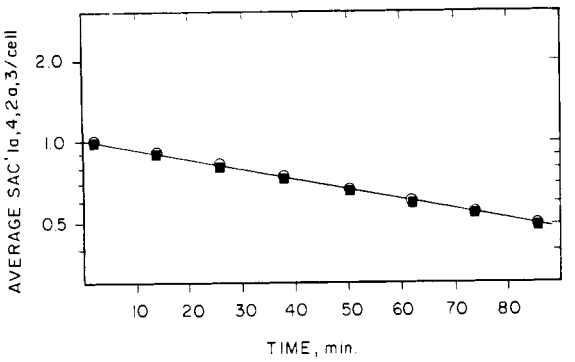

FIGURE 1: Decay of SAC'4,2a,3 in buffer C^{+} with or without ATEe (0.01 M) as indicated. (■—■) Control and (○—○) 0.01 M ATEe.

Inhibitor solutions were made freshly every day and used as soon as possible.

Removal of Inhibitor from C'3 by Dialysis. A precisely measured volume of solution containing C'3 and inhibitor was placed in a collodion bag (Schleicher & Schuell, Keene, N. H.) and dialyzed overnight at 3° against two changes of about 1000 volumes of buffer C^{2+}. The sample was then transferred from the collodion bag into a weighed tube, followed by two washings with several drops of buffer in order to assure complete recovery. The tube was then weighed again to determine its content (specific gravity of one was assumed for conversion of weight into volume). In control experiments it was found that 95 ± 1% of C'3 activity could be recovered after dialysis.

Agglutination of EAC'4,2a,3 by Anti-C'3. Rabbit antiguinea pig C'3 was prepared as described in the preceding paper (Shin and Mayer, 1968a). Agglutination tests were performed in 12 × 74 mm test tubes by mixing 0.25 ml of EAC'4,2a,3 (3×10^7 cells per ml) with 0.25 ml of various antibody dilutions in buffer B. The tests were read by inspection of the pattern of settling after 3 hr at room temperature and 10 hr at 3°.

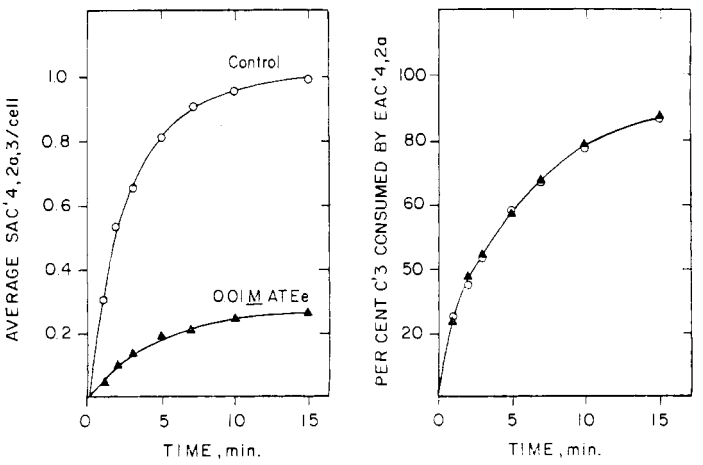

FIGURE 2: Formation of SAC'4,2a,3 and consumption of C'3 by EAC'4,2a in the presence or absence of ATEe (0.01 M) as indicated. (○) Control and (■) 0.01 M ATEe.

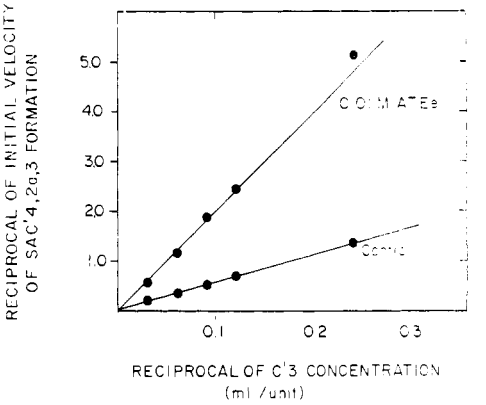

FIGURE 3: Double-reciprocal plot of initial velocity of SAC'4,2a,3 formation expressed as (minutes × cell) per average SAC'4,2a,3 on ordinate against C'3 concentration expressed as milliliters per unit on abscissa. Upper line in the presence of 0.01 M ATEe and bottom line without inhibitor.

Results

Effect of ATEe[1] *on C'3.* C'3 (10 units/ml) was mixed with an equal volume of 0.02 M ATEe and incubated for 2 hr at 25°. The reaction mixture was dialyzed and C'3 activity was assayed. No loss of C'3 activity was noted.

Decay of EAC'4,2a,3 in the presence of ATEe. Ice-cold EAC'4,2a,3 (1 ml) (1.54×10^9 cells per ml) with a low number of SAC'4,2a,3 sites per cell was incubated at 24° with 9 ml of prewarmed buffer containing 0.01 M ATEe. At regular intervals, 0.25-ml samples were pipetted into 2.5 ml of ice-cold buffer for assay of the decay rate. The diluted samples were centrifuged, washed once, drained carefully, and the cells were resuspended in 0.25 ml, followed by treatment with 1.0 ml of converting reagent A. A control experiment was performed in the absence of ATEe. The results, shown in Figure 1, indicate that the decay rate is not affected by ATEe.

Effect of ATEe on the Formation of EAC'4,2a,3 and Destruction of C'3. EAC'4,2a (1.54×10^8 cells per ml) were incubated at 25° with an equal volume of C'3 (40 units/ml) in the presence of ATEe at a final concentration of 0.01 M. There was 75% inhibition of the formation of SAC'4,2a,3 at 15 min compared with a control without ATEe. On the other hand, the consumption of C'3 from the fluid phase was exactly the same with or without inhibitor, as shown in Figure 2. These observations suggest that the initial interaction of C'3 with EAC'4,2a is not affected by the inhibitor, but a step leading to the fixation of C'3 is blocked.

Nature of Inhibition. EAC'4,2a were incubated at 24° with an equal volume of C'3, ranging between 4.2 and 33 units per ml, in the presence of ATEe at a final concentration of 0.01 M. The initial velocity of formation of SAC'4,2a,3 was measured by the method described in the preceding paper (Shin and Mayer, 1968b). A plot of the reciprocal of initial velocity of SAC'4,2a,3 for-

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: ATEe, *N*-acetyl-L-tyrosyl ethyl ester. For other abbreviations, see the preceding two papers.

mation against the reciprocal of C'3 concentration is shown in Figure 3 for the experiment with ATEe as well as the control. The result is difficult to interpret because the procedure lacks the precision of the one-step titration and because the measurement of SAC'4,2a,3 formation, unlike that of C'3 consumption, cannot be studied in the region of large C'3 excess.

Effect of Concentration of ATEe on SAC'4,2a,3 Formation. C'3 was incubated with EAC'4,2a at 24° for 10 min in the presence of ATEe at concentrations ranging from 0.0005 to 0.016 M. As shown in Figure 4, the extent of inhibition increased with concentration of inhibitor, but at a progressively diminishing rate.

Effect of Other Inhibitors. EAC'4,2a (1.54×10^8 cells per ml) were incubated for 10 min at 24° with an equal volume of C'3 (40 units/ml) in the presence of various inhibitors at final concentrations of 0.001 M. The formation of EAC'4,2a,3 and depletion of C'3 were studied. For purposes of control, EAC'4,2a, EAC'4,2a,3, and C'3 were separately incubated for 30 min at 24° with inhibitor at a final concentration of 0.001 M. The decay rate of the intermediates and the stability of C'3 were assayed. Complete analyses were made only on the seven most potent inhibitors, as shown in Table I.

TABLE I: C'3 Inhibitors.

Compounds	% Inhibn at 0.001 M
Phloridizin	84.1
Salicylaldoxime	73.0
Catechol	57.8
N-Acetyl-L-tyrosine ethyl ester	36.5
L-Epinephrine	34.7
Resorcinol	32.2
Sodium gentisate	19.5

Phloridzin is the most potent inhibitor. Among the simpler compounds, dihydroxybenzenes and their derivatives, such as catechol and epinephrine, are effective inhibitors. Phloroglucinol, phenol, *m*-hydroxybenzaldehyde, L-tyrosine ethyl ester, *o*-hydroxybenzaldehyde, benzoic acid, and ephedrine hydrochloride also inhibited with potency in the order of citation.

Agglutination of EAC'4,2a,3 by Anti-C'3. Agglutination tests with anti-C'3 were used to determine whether the inhibitor salicylaldoxime interferes with the physical uptake of C'3 by SAC'4,2a. EAC'4,2a (1.54×10^8 cells per ml) were incubated for 30 min at 24° with an equal volume of C'3 (20 units/ml) in the presence of salicylaldoxime at a final concentration of 0.01 M. The cells were washed twice with buffer C^{2+} and suspended in the same buffer to a concentration of 1.54×10^8 cells per ml, or in buffer B to a concentration of 3×10^7 cells per ml, for the hemolytic assays or for the agglutination tests, respectively.

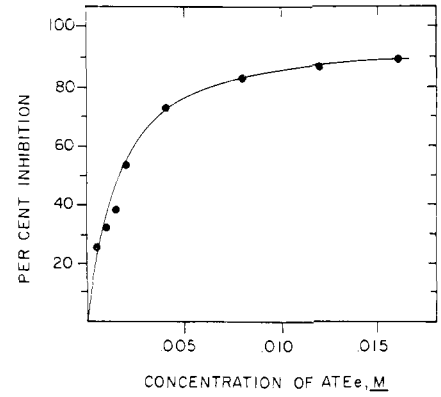

FIGURE 4: Per cent inhibition of SAC'4,2a,3 formation as a function of ATEe concentration.

The hemolytic assays indicated 98% inhibition of SAC'4,2a,3 formation in the presence of salicylaldoxime compared with a control experiment without inhibitor. Agglutination tests of the treated cells with anti-C'3 were negative at serum dilutions ranging from $^1/_{40}$ to $^1/_{640}$, as were control tests with untreated EAC'4,2a. On the other hand, the control tests in which EAC'4,2a were treated with C'3 in the absence of inhibitor, yielded cells which agglutinated with anti-C'3 up to a serum dilution of $^1/_{640}$.

Inhibition of Other Complement Reaction Steps by ATEe. One part of ice-cold EAC'1a,4,2a,3 (with a high number of SAC'1a,4,2a,3 per cell) (3.08×10^8 cells per ml) was mixed with nine parts of prewarmed C'5 (1.0 unit/ml) containing 0.001 M ATEe, and incubated at 30°. As shown in Figure 5 there was about 35% inhibition of the formation of SAC'1a,4,2a,3,5 compared with a control without ATEe.

In the same way, EAC'4,2a,3,5 were treated with C'6 (0.6 unit/ml) in the presence of ATEe at a final concentration of 0.005 M. A sample taken at 60 min showed 22% inhibition of C'6 activity, compared with a control without ATEe. Similar tests with C'7, C'8, and C'9 showed no inhibition by ATEe.

Discussion

In screening inhibitors special attention was paid to their effect on ionic strength and pH of the test systems. As reported by Nelson (1965), as well as in the preced-

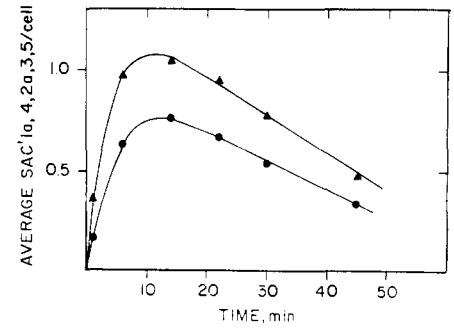

FIGURE 5: Inhibition of SAC'1a,4,2a,3,5 formation by ATEe as indicated. (▲—▲) Control and (●—●) 0.01 M ATEe.

ing article (Shin and Mayer, 1968b), ionic strength has a profound effect on the rates of the C'3, C'5, and C'6 reactions. For example, increase of ionic strength from 0.052 to 0.147 decreases the C'3 reaction rate sixfold, an average decrease of 14%/0.01 ionic strength increment. However, at the concentration used, only a few of the inhibitors had a detectable effect on ionic strength.

In the case of the C'3 reaction, ATEe and other aromatic compounds blocked only the formation of the C'3 site, but not the consumption of C'3; neither did the inhibitors inactivate native nor cell-bound C'3. This is in accord with the studies on human C'3 by Müller-Eberhard *et al.* (1966) in which it was found that phloridzin blocks the formation of the C'3 site, but not the consumption of C'3 by EAC'4,2a. Hence, the process of formation of SAC'4,2a,3 comprises more than one step, as shown also, on different grounds, in the preceding paper (Shin and Mayer, 1968b).

In an effort to elucidate the significance of the inhibition of the C'3 reaction, we studied the subsequent reaction steps in exploratory experiments which showed that ATEe also blocks the reactions with C'5 and C'6. Therefore, we have been led to consider the possibility that the C'3, C'5, and C'6 reactions may constitute a unit in a mechanistic sense. Support for this hypothesis can be derived also from another experiment which indicates that cell-bound C'2a is required for the reactions involving C'3, C'5, and C'6 (Inoue and Nelson, 1966; H. S. Shin, R. J. Pickering, and M. M. Mayer, to be published). Further studies on the validity of this concept are in progress.

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