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# STUDIES ON THE THIRD COMPONENT (C'3) OF GUINEA PIG COMPLEMENT[1]

## II. DECAY AND GENERATION OF SAC' 1a,4,2a,3

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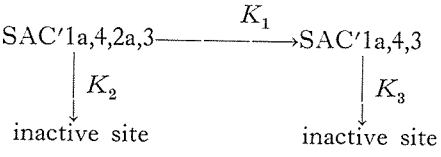
SUMMARY Decay and generation curves of SAC'1a,4,2a,3 under various conditions were analyzed. SAC'1a,4,2a,3 seems to decay via two alternative routes as following:




where SAC'1a,4,3 can be reactivated to SAC'1a,4,2a,3 by reacting with C'2.

Decay of IA reactivity of guinea pig C'3 in the intermediate complex was also demonstrated.

## INTRODUCTION

Since the discovery of human $\beta_{1C}$ globulin by MÜLLER-EBERHARD and his coworkers (1960) C'3 has been extensively studied about its conversion to an inactive form ($\beta_{1A}$), which has a different mobility on electrophoresis and is formed by aging or by interaction with the immune complex fixed with early-reacting complement components. It has also been demonstrated that there are several antigenic determinants on the C'3 molecule and that the molecule can be split into different fragment molecules (WEST *et al.*, 1966; MAYER *et al.*, 1967).

The C'3 step in immune hemolysis, however, has not yet been thoroughly analyzed, although decayed cells from EAC'1a, 4, 2a, 3 are known to be reactivated by C'2 (NISHIOKA and LINSCOTT, 1963; INOUE in RAPP and BORSOS, 1963). This paper reports an analysis of the decay and generation processes of SAC'1a, 4, 2a, 3.

## THEORETICAL ANALYSES

a) *Decay of SAC' 1a, 4, 2a, 3*

On the basis of reactivation of the decayed

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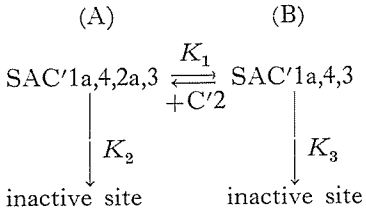
product of SAC'1a, 4, 2a, 3 on addition of C'2 (NISHIOKA and LINSCOTT, 1963, INOUE in RAPP and BORSOS, 1963), the following decay routes can be supposed.




where

$A$=average number of SAC'1a, 4, 2a, 3 per cell at time, $t$,

$A_0$=initial value of $A$,

$B$=average number of SAC'1a, 4, 3 per cell at time, $t$,

$B_0$=initial value of $B$,

$K_1$=specific rate constant of decay of SAC'1a, 4, 2a, 3 to SAC'1a, 4, 3,

$K_2$=specific rate constant of decay of SAC'1a, 4, 2a, 3 to an inactive site without passing through the SAC' 1a, 4, 3 site

$K_3$=specific rate constant of decay of SAC'1a, 4, 3 to an inactive site.

The equation will be:

$$\frac{dA}{dt} = -(K_1+K_2)A \tag{1}$$

$$\frac{dB}{dt} = K_1A-K_3B \tag{2}$$

On integration, one obtains:

$$A = A_0e^{-(K_1+K_2)t}, \tag{3}$$

$$B = B_0e^{-K_3t}+\frac{K_1A_0}{K_1+K_2-K_3} \times[e^{-K_3t}-e^{-(K_1+K_2)t}] \tag{4}$$

$$A+B = B_0e^{-K_3t}+\frac{A_0}{K_1+K_2-K_3} \times[(K_2-K_3)e^{-(K_1+K_2)t}+K_1e^{-K_3t}]. \tag{5}$$

Equation (4) is not applicable when

$$K_1+K_2 = K_3.$$

For this special case:

$$B = (B_0+K_1A_0t)e^{-K_3t} \tag{6}$$

$$A+B = (A_0+B_0+K_1A_0t)e^{-K_3t} \tag{7}$$

Experimentally, one can estimate only $A$ and $(A+B)$. The value $A$ can be obtained by lyzing intermediate cells containing the site of SAC'1a, 4, 2a, 3 by a reagent supplying sufficient C'5, C'6, C'7, C'8 and C'9. The value $(A+B)$ can be obtained by lyzing cells containing SAC'1a, 4, 2a, 3 and/or SAC'1a, 4, 3 by a reagent supplying sufficient C'2, C'5, C'6, C'7, C'8 and C'9.

Furthermore, if there are sufficient free C'2 molecules in the medium to push SAC'1a, 4, 3 back to SAC'1a, 4, 2a, 3 one can obtain the value of $A'$ formed exclusively by route $K_2$.

In this case ($K_1=0$):

$$A' = A_0e^{-K_2t} \tag{8}$$

The average number of intermediate sites can be calculated by MAYER's formula (1961):

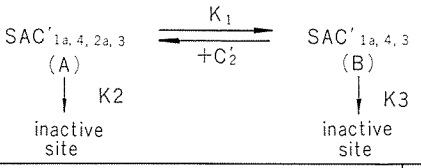
$$z = -\ln(1-y),$$

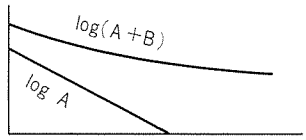
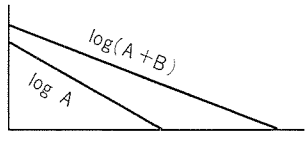
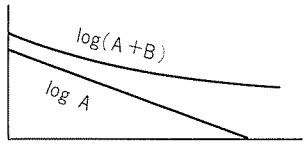
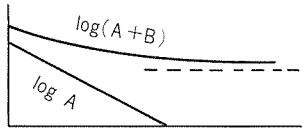
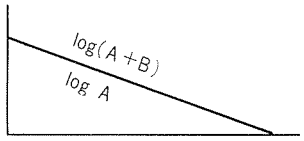
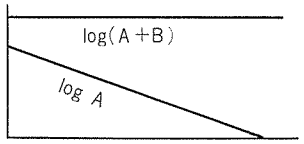
where

$y$=the proportion of cells in a particular intermediate state estimated by the particular reagent.

If any one of these three routes, $K_1$, $K_2$ and/or $K_3$ is absent, equations (3) and (5) are converted as shown in Fig. 1. If one plots the values log $A$ and log $(A+B)$ against time, $t$, the curves shown in the same figure should be obtained in the respective cases. In practice the absolute value cannot be obtained, but only the relative value depending on the reactivities of the particular intermediate site to the reagent used. Thus, the curves, log $A$ and log $(A+B)$, in case 5, can be parallel rather than superimposed lines.

b) *Generation of SAC'1a, 4, 2a, 3 from SAC' 1a,4, 2a and C'3*

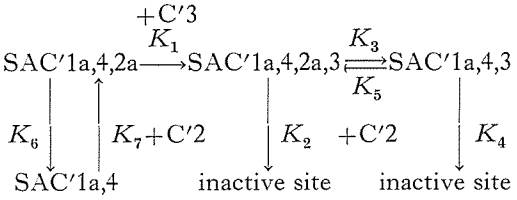



| | $K_1$ | $K_2$ | $K_3$ | A and A+B | log A and log(A+B) vs. t |
|---|---|---|---|---|---|
| 1 | + | + | + | $A_0e^{-(K_1+K_2)t}$<br>$B_0e^{-K_3t}+\frac{A_0}{K_1+K_2-K_3}\{(K_2-K_3)e^{-(K_1+K_2)}+K_1e^{-K_3t}\}$, $(K_1+K_2\neq K_3; K_2\neq K_3)$ |  |
| 2 | + | + | + | $A_0e^{-(K_1+K_2)t}$<br>$\{A_0+B_0\}\ e^{-K_2t}$<br>$(K_1+K_2\neq K_3; K_2=K_3)$ |  |
| 3 | + | 0 | + | $A_0e^{-K_1t}$<br>$B_0e^{-K_3t}+\frac{A_0}{K_1-K_3}\{K_1e^{-K_3t}-K_3e^{-K_1t}\}$<br>$(K_1\neq K_3)$ |  |
| 4 | + | + | 0 | $A_0e^{-(K_1+K_2)t}$<br>$B_0+\frac{A_0}{K_1+K_2}\{K_1+K_2e^{-(K_1+K_2)t}\}$ |  |
| 5 | 0 | + | 0 | $A_0e^{-K_2t}$<br>$A_0e^{-K_2t}$ |  |
| 6 | + | 0 | 0 | $A_0e^{-K_1t}$<br>$A_0+B_0$ |  |

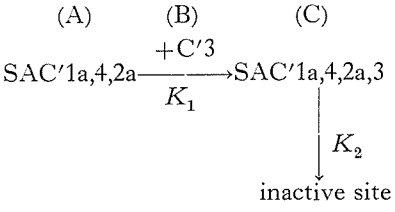
The broken line in the case 4 shows a horizontal asymptote, $z=\log\left(B+\frac{K_1}{K_1+K_2}A\right)$

FIGURE 1 Possible decay curves of SAC'1a,4,2a,3

On the basis of the known reaction routes, the following scheme is considered.




If one assumes, however, that the presence of excess free C'2 molecules in the reaction medium maintain the sites, SAC'1a, 4 and SAC'1a, 4, 3 as SAC'1a, 4, 2a and SAC'1a, 4, 2a, 3, respectively, the above scheme can be simplified as follows:




From this scheme equations identical to those given for SAC'1a, 4, 2a formation from SAC' 1a, 4 and C'2 by BORSOS *et al.* (1961) are obtained. Therefore, if the assumption about the immediate saturation of SAC'1a, 4 and SAC' 1a, 4, 3 by C'2 from the reaction medium is actually true, the number of SAC'1a, 4, 2a, 3 formed should be proportional to the concentration of C'3 used at any time during the experiment; and *t max* should be determined by the average number of SAC'1a, 4, 2a, i.e., the average number of SAC'1a, 4 in this case, and it should be independent to the concentration of C'3 used.

## MATERIALS AND METHODS

The materials and methods used were described in the first paper of this series (YONEMASU and INOUE, 1968).

## RESULTS

### 1. *Decay of SAC'1a, 4, 2a, 3*

EAC'1a,4 cells (*t max* for SAC'1a,4,2 generation at a cell concentration of $7.5 \times 10^7$/ml (BORSOS *et al.*, 1961)=7 min) were allowed to react with partially purified C'2 at a concentration of $1.5 \times 10^8$ cells/ml and about 200 effective molecules of C'2 per cell at 30°C for 4 min in VB-saline. After incubation most of the reaction mixture was poured into a flask containing an equal volume of purified C'3 preparation #9 diluted with VB-saline, and a little of the reaction mixture was introduced into VB-saline to serve as control EAC'1a, 4, 2a cells. Both mixtures were shaken continuously at 30°C for 9 min. Then the reaction mixtures were both diluted with a large volume of cold VB-saline and centrifuged. The cells were washed twice with VB-saline in the cold and resuspended in the same buffer at a concentration of $1.0 \times 10^9$ cells/ml.

At time, 0, the EAC'1a,4,2a,3 cells were pipetted into a flask containing 19 volumes of VB-saline (flask P) or partially purified C'2, supplying about 500 effective molecules of C'2 per cell (flask Q) prewarmed at 30°C.

The reaction mixtures were incubated at 30°C with continuous shaking. At intervals a 1.0 ml aliquot was transferred from flask P into a tube containing 1.5 ml of cold C'5–9 reagent supplying moderate excesses of C'5, C'6, C'7, C'8 and C'9 in 0.01 M EDTA-VB-saline (series R), and incubated at 37°C for 90 min, or to a tube containing 0.5 ml of cold partially purified C'2 supplying about 200 effective molecules of C'2 per cell in VB-saline (series S). After incubation at 30°C for 10 min, the tubes of series *S* received 1.0 ml of C'5–9 reagent supplying the same amounts of C'5, C'6, C'7, C'8 and C'9 as in series *R* in the same volume of 0.01 M EDTA-VB-saline and the tubes were incubated further at 37°C for 90 min. After incubation, the reaction mixtures from both series were diluted with 5.0 ml of cold physiological saline and centrifuged. The optical density of the supernatants was read at 413 m$\mu$.

From flask Q a 1.0 ml aliquot was transferred by pipetted to a tube (series T) containing 1.5 ml of C'5–9 reagent in 0.01 M EDTA-VB-saline as in series *R*. After incubation at 37°C for 90 min the reaction mixture was diluted with 5.0 ml of physiological saline and centrifuged. The optical density of the supernatant was read at 413 m$\mu$.

From flask Q 1.0 ml aliquots were also transferred at intervals to tubes containing 4.0 ml of cold VB-saline (series U) and tubes were centrifuged immediately. Residual C'2 in the supernatant was measured by BORSOS' method (1961).

Aliquots of 1 ml of cell suspension from each flask were also transferred to 1.5 ml of VB-saline or distilled water and served as controls of cells and completely lyzed cells. One ml of EAC'1a,4,2a cells at a concentration of $1.0\times10^8$ cells/ml was treated with C'5–9 reagent or C'2 and then the C'5–9 reagents as other controls (CBC controls).

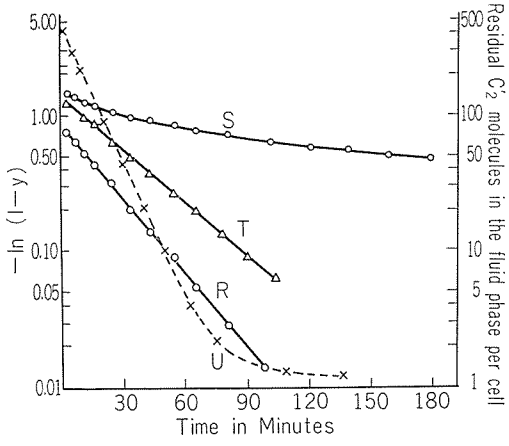



FIGURE 2 Decay of SAC'1a,4,2a,3

(R) Decay of SAC'1a,4,2a,3 without free C'2

(S) Decay of total sites of SAC'1a,4,2a,3 and SAC'1a,4,3

(T) Decay of SAC'1a,4,2a,3 in excess free C'2

(U) Residual C'2 activity of reaction mixtures series (T)

For experimental conditions and procedures, see text.

As shown in Fig. 2, the decay of SAC'1a, 4, 2a, 3 (curve $R$) proceeds exponentially as expected from equation (3) and the half-life was 16.5 min.

The shape of curve $S$ corresponds to the curve of log $(A+B)$ in either case 1 or 3 of Fig. 1, because the rate of decrease of the sum of the two kinds of site slows down but does not show any tendency to stop. Thus at least, the routes of $K_1$ and $K_3$ exist.

The experiment shown by curve T, in which the excess free C'2 present kept pushing the decayed site, SAC'1a, 4, 3 back to SAC'1a, 4, 2a, 3, gave a straight line on a semilogarithmic scale which was not parallel to curve $R$. Curve $T$, in fact, shows a half-life of 24 min. Furthermore, during the experimental period, the concentration of free C'2 in the reaction medium was maintained at sufficiently high a level to react with the SAC'1a, 4, 3 sites, although it was destroyed fairly fast, probably due to the action of C'1a on the cells (curve $U$). Therefore, curve $T$ corresponds to equation (8), indicating the possible existence of the $K_2$ route, if we suppose that the reaction from SAC'1a, 4, 3 to SAC'1a, 4, 2a, 3 by C'2 is faster than the decay of the SAC'1a, 4, 2a, 3 to SAC'1a, 4, 3. Other experiments using EAC' 1a, 4, 2a, 3 with different starting numbers of SAC'1a, 4, 2a and SAC'1a, 4, 2a, 3 always gave straight lines of decay with half-lives of 23 to 25 min, indicating the possible saturation of SAC'1a, 4, 2a, 3. Moreover, if the reverse reaction of SAC'1a, 4, 3 to SAC'1a, 4, 2a, 3 by C'2 occurs merely to slow down the reaction by the $K_1$ route and the $K_2$ route does not exist, the curve $T$ should bend as the amount of free C'2 decreases to below the level regarded as a constant excess for regeneration of SAC'1a, 4, 2a, 3, as shown in Fig. 2.

### 2. *Generation of SAC'1a, 4, 2a, 3 from SAC' 1a, 4, 2a and C'3 in the presence of excess free C'2*

EAC'1a,4 cells ($t$ *max*=6 min) at a concentration of $3.0\times10^8$ cells/ml in VB-saline were allowed to react with an equal volume of partially purified C'2 diluted in VB-saline, supplying about 370 effective molecules of C'2 per cell, at 30°C for 4 min. At time, 0, a portion of the reaction mixture was introduced into a flask containing two volumes of a 1/3,000, 1/6,000 or 1/12,000 dilution of purified C'3 preparation #9 in VB-saline prewarmed at 30°C. The reaction mixtures were incubated at 30°C with continuous shaking.

At intervals a 1.0 ml aliquot was taken from each flask and pipetted into a tube containing 10 ml of cold VB-saline, and centrifuged in the cold. The supernatant was discarded, and tube was drained

and wiped with filter paper. The sedimented cells were then resuspended in 1.0 ml of VB-saline and mixed with 1.5 ml of a C'5–9 reagent in 0.01 M EDTA-VB-saline. The tube was incubated at 37°C for 90 min. After incubation, 5.0 ml of cold physiological saline were added and the optical density of the supernatant was read at 413 mμ.

Controls of cells and completely lyzed cells were included with each reaction system.

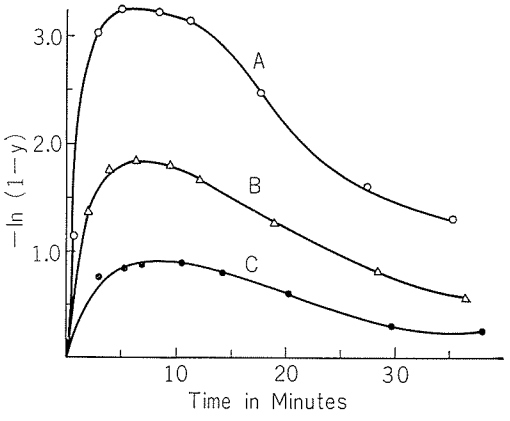



FIGURE 3 Generation of SAC'1a,4,2a,3 from SAC' 1a,4,2a and C'3 in the presence of excess free C'2 C'3 concentration: 1/4,500 (A), 1/9,000 (B) and 1/18,000 (C).

As shown in Fig. 3, the number of SAC'1a, 4, 2a, 3 sites formed at any moment was approximately proportional to the concentration of C'3 used. The peak time of formation of the sites was independent of the concentration of C'3. These results are as predicted supposing that the number of SAC'1a, 4, 2a is maintained in constant large excess over C'3 by the free C'2 added, and the results are similar to those obtained for SAC'1a, 4, 2a formation from SAC'1a, 4 and C'2 by BORSOS *et al.* (1961)

At the end of the experiment (at t=66 to 68 min), an aliquot of each reaction mixture was centrifuged and free C'2 remaining in the supernatant of the reaction medium was estimated. It was observed that 1.7 to 2.0 effective molecules of C'2 per cell remained in the medium.

Two batches of EAC'1a,4 cells (*t max*=5 min and 7 min, respectively) were used for preparing EAC'1a, 4,2a. The experiment procedures used were similar to those in the previous experiment using both batches of cells and two-fold dilutions of purified C'3.

As shown in Fig. 4, the time of the peak in the number of SAC'1a, 4, 2a, 3 depended on the EAC'1a, 4, 2a cells used, and was independent of the C'3 concentration. These results are also similar to those of BORSOS *et al.* (1961).

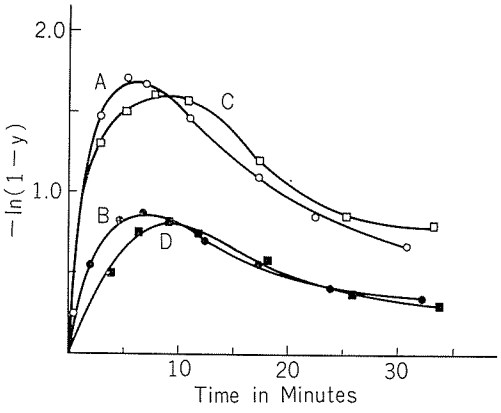



FIGURE 4 Generation of SAC'1a,4,2a,3 from SAC' 1a,4,2a and C'3 in the presence of excess free C'2 *t max* of EAC'1a,4: 5 min for A and B, 7 min for C and D; C'3 concentration: 1/10,000 for A and C, 1/20,000 for B and D.

### 3. *Generation of SAC'1a, 4, 2a, 3 from cells with excess SAC'1a, 4, 2a and limited C'3*

EAC'1a,4,2a cells were prepared from EAC'1a,4 (*t max*=6 min) and partially purified C'2, at a concentration of about 200 effective molecules of C'2 per cell, in VB-saline, at 30°C. The cells were centrifuged, and washed and resuspended in VB-saline at a concentration of $4.0 \times 10^8$ cells/ml.

The cells were pipetted into a flask containing 7 volumes of a prewarmed 1/3,500 (A) or 1/10,500 dilution (B) of purified C'3 preparation in VB-saline. The reaction mixtures were incubated at 30°C with continuous shaking. At intervals, a 1.0 ml aliquot was transferred to a tube containing 10 ml of cold VB-saline, and centrifuged. The supernatant was discarded, and the tube was drained and wiped with filter paper. The sedimented cells were resuspended in 1.0 ml of cold VB-saline and 1.5ml of a C'5–9 reagent was added. After incubation at 37°C for

90 min, 5.0 ml of cold physiological saline were added and the mixture was centrifuged. The opitcal density of the supernatant was measured at 413 mμ. Control cells and completely lyzed cells and EAC'1a, 4,2a cells with C'5–9 reagent were also included.

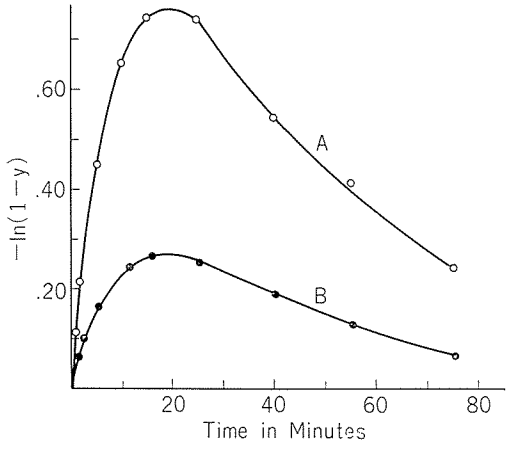



FIGURE 5 Generation of SAC'1a,4,2a,3 from cells with excess number of SAC'1a,4,2a and limited C'3
C'3 concentration: 1/4,000 (A) and 1/12,000 (B)

As shown in Fig. 5, the shapes of the SAC' 1a, 4, 2a, 3 generation curves were similar to those obtained in the previous experiment, but the amount of SAC'1a, 4, 2a, 3 formed was not proportional to the concentration of C'3 used. This seems to reflect the complicated decay route of the intermediate complexes involved.

4. *Generation of SAC'1a, 4, 2a, 3 from a large excess of C'3 and limited number of SAC'1a, 4, 2a on the cell.*

EAC'1a,4,2a cells were prepared from many EAC' 1a,4 cells and a limited amount of partially purified C'2 in VB-saline. The cells were centrifuged, washed twice with VB-saline and resuspended in the same buffer at a concentration of $2.0 \times 10^8$ cells/ml. The cells were prewarmed at 30°C, and then mixed with three volumes of purified C'3 diluted in VB-saline and also prewarmed at 30°C, supplying about 1,000 molecular units of C'3 per cell judged by the C'5–9 reagent used. The reaction mixture was incubated at 30°C with continuous shaking.

At intervals a 1.0 ml aliquot was transferred to a tube containing 10 ml of cold VB-saline, and the mixture was centrifuged. The supernatant was discarded, and the tube was drained and wiped with filter paper. The cells were resuspended in 1.0 ml of cold VB-saline and 1.5ml of a C'5–9 reagent in

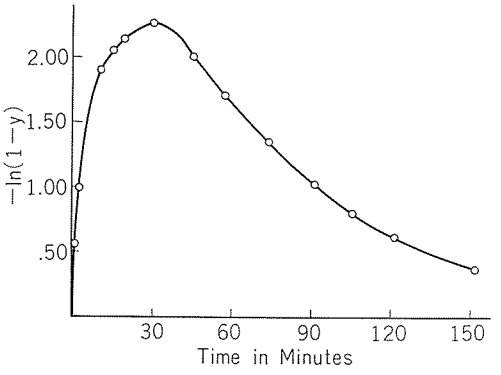



FIGURE 6 Generation of SAC'1a,4,2a,3 from large excess of C'3 and limited number of SAC'1a,4,2a on the cell

For experimental conditions and procedures, see text.

0.01 M EDTA-VBSS was added. After incubation at 37°C for 90 min, 5.0 ml of cold physiological saline were added and the mixture was centrifuged. The optical density of the supernatant was measured at 413 mμ.

As shown in Fig. 6, the shapes of the generation curves were similar to those obtained in previous experiments. Although theoretical analysis is difficult, the results also show the existence of the decay route(s) of SAC'1a, 4, 2a, 3.

5. *Generation of SAC'1a, 4, 2a, 3 from SAC' 1a, 4, 3 and large excess of C'2*

EAC'1a,4,2a,3 cells were prepared from EAC'1a,4 cells ($t\ max = 7.5$ min) and moderate excess of partially purified C'2 and a limited amount of purified C'3 in VB-saline. The cells were centrifuged, washed twice with, and resuspended in the same buffer. They were incubated at 37°C for 60 min to allow decay. The cells were then centrifuged and washed once with, and resuspended in VB-saline at a concentration of $1.0 \times 10^8$ cells/ml.

At time, 0, the cell suspension, prewarmed at 30°C

for 15 min, was poured into a flask (A) containing an equal volume of partially purified C'2 diluted in VB-saline, supplying about 500 effective molecules per cell, also prewarmed at 30°C for 15 min. A control system (B) containing VB-saline only instead of C'2 was included. The reaction mixtures were incubated at 30°C with continuous shaking.

At intervals, a 1.0 ml aliquot was transferred to a tube containing 10 ml of cold 0.01 M EDTA-VBSS and the mixture was centrifuged. The supernatant was discarded, and the tube was drained and wiped with filter paper. The cells were resuspended in 1.0 ml of VB-saline and 1.5 ml of a C'5–9 reagent in 0.01 M EDTA-VBSS was added. Control cells and completely lyzed cells were included. The reaction mixture was incubated at 37°C for 90 min with continuous shaking. After incubation 5.0 ml of physiological saline were added and the mixture was centrifuged. The optical density of the supernatant was measured at 413 m$\mu$.

As shown in Fig. 7, C'2 can reactivate decayed site, SAC'1a, 4, 3 to SAC'1a, 4, 2a, 3. The generation curve also rises to a maximum, and after a peak value the number of SAC'1a, 4, 2a, 3 decreases even in the presence of free C'2 in the reaction medium. If the decay route of $K_2$ in Fig. 1 is absent, i.e., if the decay occurs only through SAC'1a, 4, 3, the curve should approximate to a plateau in the steady-state between SAC'1a, 4, 3 and excess free C'2, if decay to an inactive site from transient SAC' 1a, 4, 3 formed even in the presence of free C'2, is negligible.

The increase in the difference between curves $S$ and $R$ shown in Fig. 2 indicates that the decay through the $K_3$ route is relatively slow. Therefore, the result obtained in this experiment also suggests the presence of a decay route from SAC'1a, 4, 2a, 3 to an inactive site without passing through the state of SAC'1a, 4, 3.

### 6. *Decay of IA reactivity of C'3 in the intermediate complexes, SAC' 4, 2a, 3 and SAC' 4, 3*

EAC'1a,4,2a cells were prepared from EAC'1a,4 ($t$ *max*=6 min) and partially purified C'2 in VB-saline at a ratio of about 500 effective C'2 molecules per cell. The cells were centrifuged and washed twice with 0.01 M EDTA-VB-saline. The resulting EAC'4, 2a cells were resuspended in the same buffer at a concentration of $5.0\times10^7$ cells/ml.

The purified C'3 preparation #20 was diluted with 0.01 M EDTA-VB-saline to obtain serial two-fold dilutions from 1/200 to 1/204,800 in test tubes. One drop of each dilution was delivered into a cup in the first row of each of three Microtiter plates,as shown in Table 1. As a control, the last cup of the row received one drop of 0.01 M EDTA-VB-saline instead of C'3. All cups in this row received a drop of EAC'4, 2 cells at a concentration of $5.0\times10^7$ cells/ml. Two (B and C) of the three plates were sealed with Scotch tape and all three plates were incubated at 30°C for 20 min with continuous shaking. Plate B was stored in a refrigerator for 22 hr. Plate C was stored at 37°C for the same period. (This concentration of EDTA is known to prevent the growth of contaminant microbe.) On the last plate (A) the cells in each cup in the first row were diluted immediately with 0.01 M EDTA-VB-saline in the cross direction of the row as shown in Table 1, using

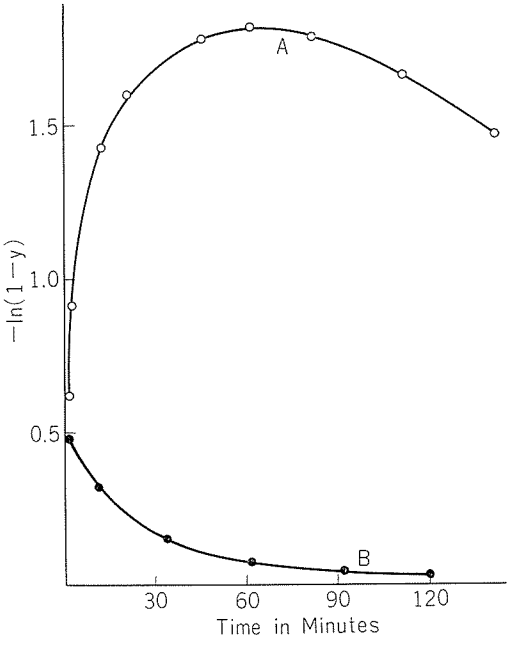



FIGURE 7 Generation of SAC'1a,4,2a,3 from SAC' 1a,4,3 and large excess of C'2
(A) SAC'1a,4,3 in large excess of C'2
(B) SAC'1a,4,3 in VB-slaine instead of C'2
For experimental conditions and procedures, see text.

diluting loops. The last row was not diluted and served as controls. All cups received a drop of human erythrocytes, type O, at a concentration of $1.0 \times 10^8$ cells/ml. The plate was vibrated at 37°C for 15 min and then stood at 37°C for 60 min before reading.

Plates B and C were treated in the same way on the next day.

As shown in Table 1, the IA reactivity of C'3 fixed on the intermediate cells had clearly decayed after 22 hrs storage even in the cold, although NISHIOKA reported that he could demonstrate no decay of IA reactivity of either guinea pig or hunan C'3 (1968).

## DISCUSSION

NISHIOKA and LINSCOTT (1963) showed that C'2 can reactivate the hemolytic reactivity of the decayed cells from EAC'1a, 4, 2a, 3. INOUE analyzed this decay process and suggested the possible existence of two alternative routes to the inactive sites (in RAPP and BORSOS, 1963).

From curve *R* in Fig. 2, SAC'1a, 4, 2a, 3

TABLE 1 *Decay of IA reactivity of C'3 in the intermediate complexes, SAC'4, 2a, 3 and SAC'4, 3*

| Expt. | Concentration of cells at dilution (cells/ml) | Concentration of C'3 added to the 1st row | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 : 200 | 1 : 400 | 1 : 800 | 1 : 1,600 | 1 : 3,200 | 1 : 6,400 | 1 : 12,800 | 1 : 25,600 | 1 : 51,200 | 1 : 102,400 | 1 : 204,800 | 0 |
| A (immediate) | $5 \times 10^7$ | 4 | 4 | 4 | 3 | 3 | 3 | 3 | 2 | 2 | 1 | tr | 0 |
| | $2.5 \times 10^7$ | 4 | 4 | 3 | 3 | 3 | 3 | 2 | 1 | tr | tr | tr | 0 |
| | $1.25 \times 10^7$ | 2 | 1 | 1 | 1 | 1 | 1 | tr | tr | 0 | 0 | 0 | 0 |
| | $6.25 \times 10^6$ | 1 | tr | tr | tr | tr | tr | tr | 0 | 0 | 0 | 0 | 0 |
| | $3.13 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $1.56 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $7.81 \times 10^5$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B after 22 hr at 2–4°C | $5 \times 10^7$ | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | tr | tr | tr | 0 |
| | $2.5 \times 10^7$ | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 1 | tr | tr | 0 | 0 |
| | $1.25 \times 10^7$ | tr | tr | tr | tr | tr | tr | tr | 0 | 0 | 0 | 0 | 0 |
| | $6.25 \times 10^5$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $3.13 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $1.56 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $7.81 \times 10^5$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C after 22 hr at 37°C | $5 \times 10^7$ | 2 | 2 | 2 | 2 | 2 | 1 | tr | tr | 0 | 0 | 0 | 0 |
| | $2.5 \times 10^7$ | 1 | tr | tr | tr | tr | tr | tr | 0 | 0 | 0 | 0 | 0 |
| | $1.25 \times 10^7$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $6.25 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $3.13 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $1.56 \times 10^6$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $7.81 \times 10^5$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

decays with a half-life of 16.5 min at 30°C. Curve $T$ shows that it decays with a half-life of 24 min in the presence of excess free C'2. If the assumption, mentioned in the Theoretical Analysis and in the text about this experiment, is so, the rate constants are calculated as $K_1+K_2=0.043$/min and $K_2=0.029$/min from these half-lives. $K_1$ is, therefore, calculated as the difference between them, i.e., 0.014/min. The assumption, that the $K_1$ route is blocked by the presence of excess free C'2 in the reaction mixture, is thought to be correct from the results shown in Figs. 3 and 4, where *t max* is determined only from the average number of SAC'1a, 4, 2a on the cells, and the number of SAC'1a, 4, 2a, 3 formed during the experimental period is proportional to the concentration of C'3 used. The existence of the $K_2$ route is also possible from the results of the experiement shown in Fig. 7.

If there is no $K_3$, the curve S in Fig. 2 would terminate above the horizontal asymptote $z=\log\left(Bo+\frac{K_1}{K_1+K_2}A_0\right)$; where, if one postulates $B_0=0$, the asymptote would be $z=\log\ (0.326\,A_0)$. Accordingly the initial value, $A_0$, in Fig. 2 should be more than 1.7 on extrapolation of the curve to the $z$ axis in this particular experiment, and curve $S$ should not cross the asymptote $z=\log 0.55$ when $K_3=0$. As shown in Fig. 2 curve $S$ decreases below this value. Therefore, the route of $K_3$ must also exist, although it may be very minor. These results show the existence of all the proposed routes.

The $K_1$ route corresponds to the route of decay of SAC'1a, 4, 2a to SAC'1a, 4. Therefore, both the routes of $K_2$ and $K_3$ seem to be due to degradation of active C'3 molecules on the site. Nevertheless, the rate constants $K_2$ and $K_3$ cannot be the same, for if there were the curve $S$ would be a straight line, as shown in case 2 in Fig. 1.

NISHIOKA and his coworkers could not demonstrate the decay of IA reactivity of guinea pig or human C'3 in the intermediate complex (NISHIOKA, 1968). The experiment shown in Table 1, however, show clearly the decay of IA reactivity of guinea pig C'3 activated on the intermediate site. The decay seems to correspond to the decay of the sum of SAC'1a, 4, 2a, 3 and SAC'1a, 4, 3 (A+B in Fig. 1 or curve $S$ in Fig. 2).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