STUDIES ON IMMUNE HEMOLYSIS: PREPARATION OF A STABLE AND HIGHLY REACTIVE COMPLEX OF SENSITIZED ERYTHROCYTES AND THE FIRST COMPONENT OF COMPLEMENT (EAC'1); INACTIVATION OF CELL-FIXED C'1 BY SOME COMPLEMENT REAGENTS*

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(Received for publication, August 20, 1959)

The interaction of sensitized sheep erythrocytes (EA) with complement (C') leads to hemolysis of the red cells in a sequence of several reaction steps. Levine and Mayer (1) have established that the components C'1 and C'4 first interact with the sensitized cells to form an intermediate complex EAC'1,4. Whether the transfer of hemolytically active C'1 to sensitized erythrocytes is dependent upon previous or simultaneous fixation of C'4, or whether the fixation of C'1 precedes and then mediates the subsequent action of C'4 has not been satisfactorily determined.

Pillemer and coworkers (2) were unable to transfer hemolytically active C'1 to sensitized cells in the absence of C'4. They found, after interacting hydrazine-treated complement with sensitized cells, that not only was the supernatant serum devoid of C'1 activity, but also that there was no detectable C'1 activity on the resultant cells. Becker and Wirtz (3), on the other hand, were able to obtain a reactive EAC'1 by exposing *heavily* sensitized erythrocytes to hydrazine-treated guinea pig serum. Laporte *et al.* (4) exposed an EA preparation to the euglobulin fraction (midpiece) of hydrazine-treated guinea pig serum (R4), and found that the cells were converted into EAC'1. The experiments reported by the latter two groups of investigators indicate that it is indeed possible to produce an EAC'1 in the absence of C'4 but only under certain empirically determined circumstances, the mechanisms of which are poorly understood. It appears that the interaction of hydrazine-treated guinea pig serum with sensitized erythrocytes results in deterioration of the complex EAC'1 as it is being formed. This capacity of hydrazine-treated guinea pig serum apparently can either be overcome by using heavily sensitized erythrocytes or eliminated by splitting the hemolytically active C'1 into the midpiece fraction of the R4.

* This work was supported by research grant H-1803 of the National Heart Institute of the National Institutes of Health and by the Helen Hay Whitney Foundation.

This paper reports the results of experiments on interacting sensitized sheep erythrocytes with ether-treated guinea pig serum in an effort to produce a preparation of EAC'1 highly reactive with respect to a complement reagent that contains C'2, C'3, and C'4 (R1). Also here reported are the results of an investigation of the deterioration of EAC'1 as it occurs during the interaction of sensitized sheep erythrocytes with hydrazine-treated guinea pig serum (R4). The experiments demonstrate that there is present in hydrazine-treated guinea pig serum an ether labile factor that promotes loss of the hemolytic activity of bound C'1 during its fixation in the interaction of EA with hydrazine-treated guinea pig serum.

Materials and Methods

Incubation and Centrifugation.—All incubations were performed in a water bath. In the experiments in which the incubation time was varied or in which the lysis time was studied the temperature of the reagents was adjusted and tested with a thermometer placed inside the tube before mixing the reagents. All centrifugations were carried out at 4°C.

Saline.—Buffered saline containing calcium and magnesium ions was used as recommended by Mayer *et al.* (5) but without adding bovine albumin.

Sheep Erythrocytes.—Sheep blood was collected in the abattoir in Alsever's fluid and stored in the ice box. No mixture older than 2 weeks was used.

Anti-Sheep Hemolysin.—Amboceptor was purchased[1] and its titration was carried out according to Kabat and Mayer (6). The hemolytic titer was 1600 units/ml.

Sensitization.—The principles formulated by Kabat and Mayer (6) were followed, using instead of buffered saline a physiological saline containing 0.03 M sodium citrate. After the incubation with amboceptor the cells were washed twice with citrated saline, once with buffered saline, and were then resuspended in buffered saline at a density of 0.5×10^9/ml. It was found that an optimal sensitization with regard to the titration of full complement required a minimum of 4 hemolytic units per 10^9 cells. However, in titrating C'1 with the aid of R1 no optimal sensitization could be found. The C'1 titer of active complement, R4, and ether-inactivated complement increased steadily with increasing amboceptor doses between 2 and 16 units. A sensitization dose of 8 units of amboceptor was chosen arbitrarily for most of the experiments. An additional series of experiments was carried out using cells sensitized with only 2 units of amboceptor.

Sensitized Stromata and Decomplementation.—A 100 ml. suspension of 10×10^9 sheep cells/ml. in citrated saline was mixed with an equal amount of an amboceptor dilution containing 400 units/ml. and 10 to 12 per cent glycerin. This mixture was incubated at 37° for 30 minutes. The cells were then spun down and washed thrice in citrated saline and several times in buffered saline. Cells exposed to glycerin lyse quickly once they are resuspended in glycerin-free saline. The agglutinated stromata can be spun down easily. The washing of the sensitized stromata was continued to the point at which the supernatant became colorless and the stromata showed a slightly pink color. The stromata were finally taken up in a total volume of 100 ml. of buffered saline to give a final density of 10×10^9 sensitized stromata/ml. For the purpose of decomplementation an amount of 20×10^9 sensitized stromata were suspended in 1 ml. of guinea pig serum and the suspension was incubated at 37° for half an hour. This procedure was carried out twice. It removed all C' 1 and C'4 and left variable amounts of C'2 and C'3.

[1] Carworth Laboratory, Inc., New City, New York.

Complement.—Guinea pig blood obtained by heart puncture with the animals under slight ether anesthesia was placed in centrifuge tubes coated with vaseline. The tubes were kept for 1 hour in ice water and then centrifuged in the cold. The serum was pooled and stored in sealed ampules at −40°.

Complement Reagents.—Midpiece and endpiece were obtained by dialyzing guinea pig serum in the cold against 0.02 M phosphate buffer of pH 5.4. R4 was split in the same way into R4-midpiece and R4-endpiece. R3 was obtained by treating guinea pig serum with zymosan.[2] Another R3 preparation was obtained by mixing guinea pig serum with equal volumes of a 0.5 per cent solution of cobra venom[3] and incubating the mixture for 30 minutes at 37°. R4 was obtained by incubating 1 ml. of guinea pig serum with 0.25 ml. of a 0.16 M solution of hydrazine. R1 was obtained by mixing equal parts of a 1:10 dilution of endpiece with a 1:10 dilution of H (guinea pig serum inactivated at 56° for 30 minutes). R2 was obtained by mixing equal parts of a 1:20 dilution of midpiece with a 1:10 dilution of H. R4 was used for titrations in a dilution of 1:5, R3 in a dilution of 1:30. All dilutions are in reference to the volume of the original serum. Chelation was performed by adding EDTA (ethylenediaminetetraacetate) to the reagents to give a final concentration of 0.01 M with reference to the reaction mixture.

Ether Treatment of Guinea Pig Serum.—According to Toda and Mitsuse (7) the shaking of complement with ether removes the C'4 activity. This method was studied in detail with respect to the optimal conditions required for the purposes of this investigation. It was found that the inactivation rate of C'4 is dependent upon the temperature at which the ether treatment is performed. The best procedure proved to be the following: 100 ml. of ether (ethyl oxide) are placed in a 500 ml. Erlenmeyer flask and warmed to 30° in a water bath on a magnetic stirrer. Under high speed stirring 4 ml. of guinea pig serum is added. The mixture is stirred for about 5 minutes, the ether is then rapidly decanted, and the remnants of ether removed by applying a gentle stream of compressed air into the flask for 2 or 3 minutes. The ether-treated guinea pig serum (C'-ether) is then diluted with ice-cold buffered saline 1:10 and kept in ice water. Under these conditions C'4 is inactivated completely.[4] The preparation usually shows a titer of 1600 to 3200 C'1 units and variable titers of C'2 and C'3. Freshly prepared C'-ether is markedly procomplementary, *i.e.*, it enhances the titer of whole complement; however, after 6 to 8 hours in ice water, it turns anticomplementary.

Exposure of EA to Reagents Containing C'1 and Lacking C'4.—Five centrifuge tubes in the water bath received 5 ml. each of the reagent to be tested (*i.e.*, R4). After the reagent reached the desired temperature, 5 ml. of an EA suspension of the same temperature was added quickly to each tube. The time at which the first tube received the EA suspension was marked as time zero with a stop-watch. Pipetting into the last tube was performed within less than 20 seconds from time zero. Two minutes after time zero the first tube was removed from the water bath and quickly centrifuged in the cold. The supernatant was quickly discarded and the cells were then washed thrice with ice-cold buffered saline. The time required for removing the tube from the water bath, spinning the cells down, decanting, and resuspending them was less than 90 seconds. In the same fashion tubes were removed from the water bath after 5, 10, 20, 30, and in some cases after 45 and 90 minutes, and the cells washed and resuspended. All

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[2] Zymosan, type A, Fleischman Laboratories, Standard Brands, Inc., New York.

[3] Hynson, Westcott & Dunning, Inc., Baltimore.

[4] All batches of guinea pig complement obtained and examined in New York over a period of 2 years could be readily inactivated by stirring them with ethyl oxide (U.S.P. solvent ether) for 5 minutes. All batches of guinea pig complement obtained and examined subsequently in West Germany (Düsseldorf) were found to require an exposure time of 30 to 50 minutes to ethyl oxide (Aether pro narcosi, Deutsches Arzneibuch, Berlin, 6th edition, 1926) for complete inactivation.

cell suspensions were finally readjusted to a density of 0.25×10^9 cells/ml. and kept in ice water.

Estimation of the Hemolytic Activity of Cell-Fixed C'1.—A 0.4 ml. suspension of the per-sensitized cells was placed in a Kahn tube in the 37° water bath for 5 minutes. Then 0.4 ml. of R1, which had also previously been warmed, was added quickly to the cell suspension and the stop-watch put in motion. The time required to reach 50 per cent hemolysis was estimated by inspecting the tube about every 10 seconds and comparing it with a standard hemolysate. This standard was made by mixing equal parts of an EA suspension of 0.125×10^9 cells/ml. with a chelated complement lysate from a suspension of the same density. This procedure for evaluating the lysis time, less exact than the original method devised by Becker (8), can be considered only as a semiquantitative method. This method, however, proved to be sufficiently accurate for the purpose of this investigation. The reciprocal of the 50 per cent lysis time in minutes was plotted logarithmically against the time during which the sensitized cells had been exposed to the "C'1-donor." Additionally, the degree of lysis reached after 30 minutes incubation of the cells with R1, R4, and chelated complement was estimated by comparing visually the color of the supernatant with serially diluted hemoglobin standards.

EXPERIMENTAL

An EA suspension (8 amboceptor units) was exposed at 37°C. to varying dilutions of R4 and of C'-ether. Samples were taken after an incubation time of 2, 5, 10, and 30 minutes. The cells were washed, resuspended, and examined for their 50 per cent lysis time in the presence of R1.

Fig. 1 represents the results. It can be seen that during the incubation with an R4, diluted 1:10, a considerable C'1 activity was transferred to the cells, the maximum of which was reached within the first 2 minutes. This reactivity to R1, however, decreased rapidly (curve I) and after an incubation of 5 minutes it was so reduced (50 per cent lysis time of 6 minutes) that the cells then failed to give a final lysis of 100 per cent with R1. It can be seen, furthermore, that the rate of decay of the reactivity of the cells to R1 decreased in proportion to the dilution of R4 (Fig. 1, curves II and III). This fact in itself suggests that the decay of the cell-fixed C'1 is not merely a spontaneous one but caused by the presence of R4 itself. The reactivity to R1 of the cells that had been incubated with C'-ether was practically constant during the whole incubation period and independent of the dilution at which C'-ether was used (Fig. 1, curves IV and V). Additional experiments revealed that this was true even when the mixture was incubated at 37° for 90 minutes.

The cell samples that showed the highest reactivity with R1 were tested with respect to their reactivity against R4 and chelated complement as described. They showed no detectable amount of fixed C'4 or C'2. Therefore, the cells found reactive to R1 can justifiably be regarded as corresponding to the formula EAC'1.

An experiment was performed to study the interaction of EA and the euglobulin fraction of R4 (R4-midpiece). An EA suspension (8 amboceptor units) was incubated at 37° with a dilution of 1:15 of R4-midpiece. In parallel an EA suspension was exposed to a mixture of equal parts of R4-midpiece 1:7.5 and R4-endpiece 1:7.5. The reactivity of the cells to R1 was evaluated at intervals during the incubation.

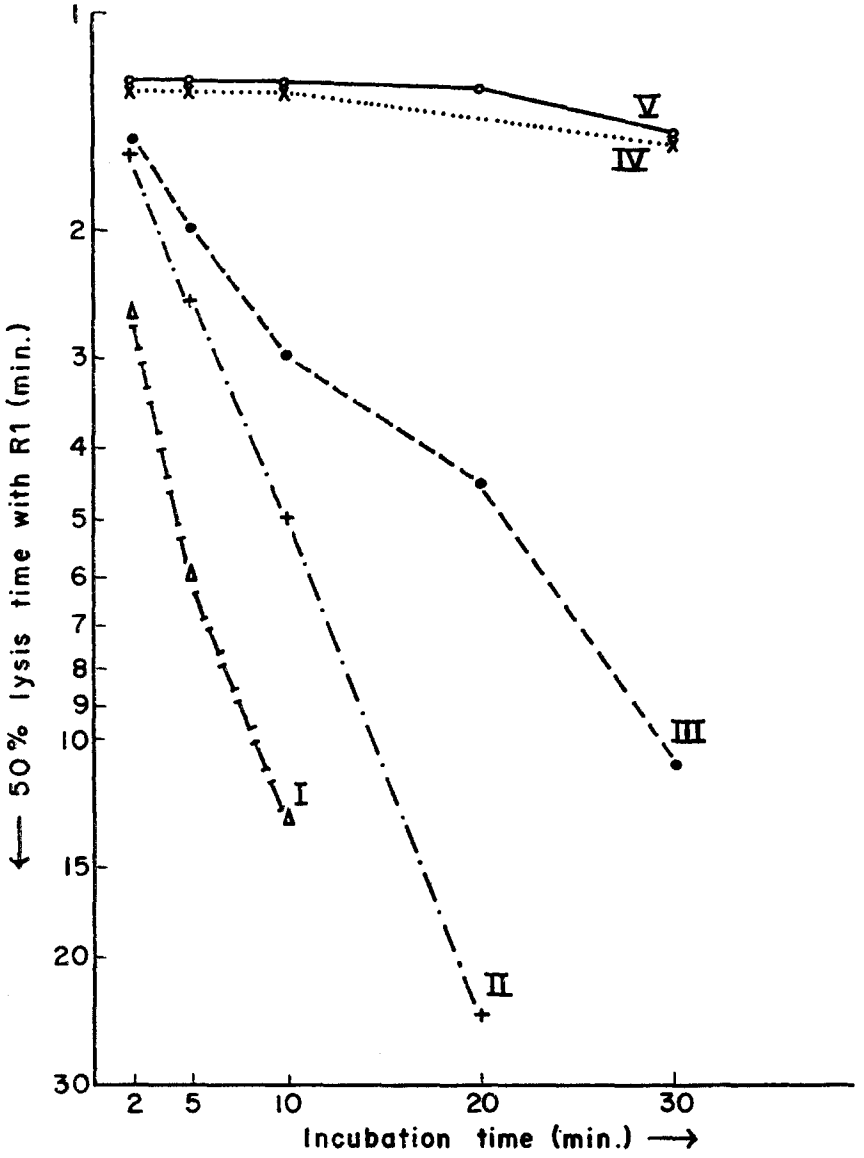

FIG. 1. Reactivity to R1 of sensitized cells during their interaction with R4 or C′-ether. I, EA plus R4 1:10. II, EA plus R4 1:20. III, EA plus R4 1:80. IV, EA plus C′-ether 1:80. V, EA plus C′-ether 1:10. All incubations performed at 37°.

Table I summarizes the results. It can be seen that when EA is exposed to the euglobulin fraction of R4 it is rapidly converted into EAC'1 and that this highly reactive compound shows no decay of its reactivity to R1 during 30 minutes of incubation. On the other hand if EA is exposed to a mixture of both fractions of R4 (euglobulin and supernatant) the resulting compound EAC'1 undergoes a rapid decay. This experiment confirms the experiments of Laporte *et al.* quoted above. It shows, furthermore, that the absence of an appreciable decay of cell-fixed C'1 in the reaction mixture EA plus R4-midpiece is not due to some hypothetical change of R4 during its dialysis at pH 5.4. The fact that the inactivation occurs in the presence of R4-endpiece shows that the splitting of R4 into midpiece and endpiece merely separates the decay inducing property

TABLE I

Reactivity to R1 of an EAC'1 Prepared from R4-Midpiece

Incubation time of the reaction mixture	Reaction mixture I: EA plus R4-midpiece		Reaction mixture II: EA plus R4-midpiece plus R4-endpiece	
	50 per cent lysis time with R1	Lysis after 30 min. exposure to R1	50 per cent lysis time with R1	Lysis after 30 min. exposure to R1
min.	*min.*	*per cent*	*min.*	*per cent*
2	1.25	100	2	100
5	1.25	100	2.75	100
10	1.25	100	7	90
20	1.25	100	9	80
30	1.25	100	30	50

from the complement component C'1, the former being located in the supernatant and the latter being precipitated with the euglobulins.

An experiment was performed to demonstrate the influence of temperature upon the formation and the decay of EAC'1 in the reaction mixture EA plus R4. An EA suspension (8 amboceptor units) was mixed with equal parts of a 1:20 dilution of R4 and incubated at 3°, 13°, 25°, and 37°. Samples were taken after 2, 5, 10, 20, and 30 minutes and examined for their reactivity to R1.

Fig. 2 shows that regardless of the temperature the peak of reactivity was reached in less than 2 minutes of exposure time. The subsequent decay of the cell reactivity was most marked at 37°, whereas it progressed slowly though perceptibly at an incubation temperature of 3°.

The decay of EAC'1 in the mixture R4 plus EA could be attributed either to the hydrazine itself which is present, though diluted, in R4, or to a product of the interaction of hydrazine with serum constituents. To examine what role the hydrazine plays in the decay of EAC'1 exposed to R4, several experiments were performed.

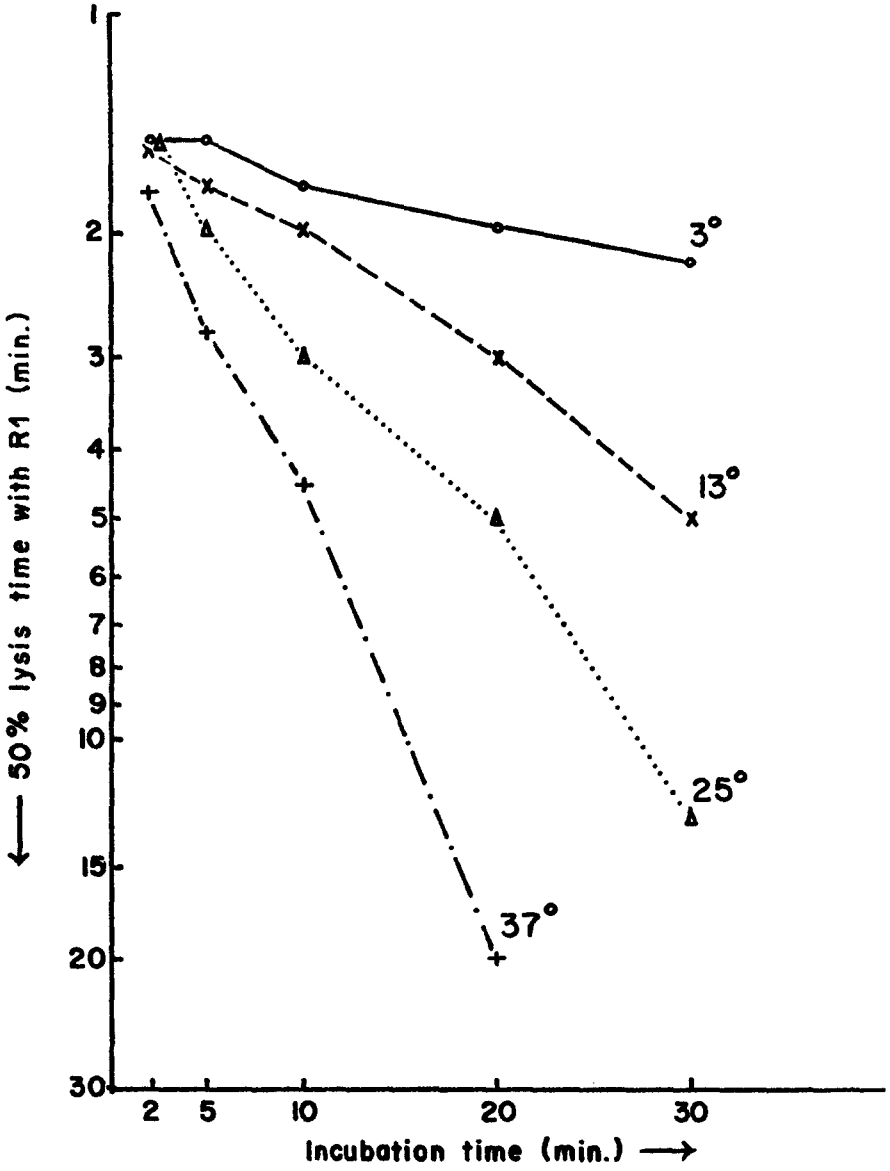

FIG. 2. Effect of the incubation temperature on the hemolytic reactivity of sensitized cells to R1 during their exposure to R4.

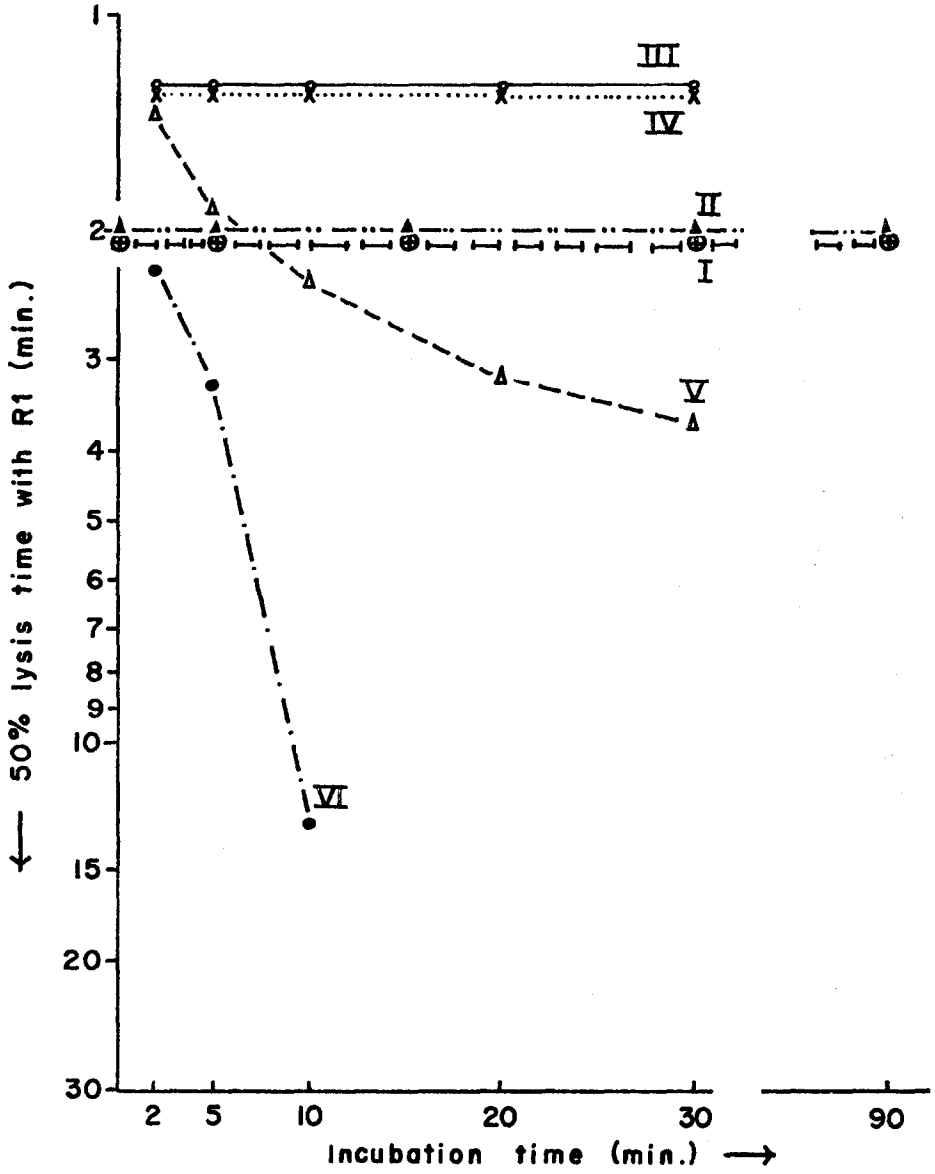

FIG. 3. Cell reactivity to R1 during an incubation at 37°. I, EAC'1 in saline. II, EAC'1 in saline plus hydrazine 0.016 M. III, EA plus ether-treated complement. IV, EA plus complement treated first with ether and then with hydrazine. V, EA plus complement treated first with hydrazine and then with ether. VI, EA plus hydrazine treated complement.

A preparation of EAC'1 was obtained by exposing EA (8 amboceptor units) to a 1:80 dilution of R4 at 3° for 5 minutes. One series of samples of this EAC'1 preparation was incubated at 37° with buffered saline containing 0.016 M hydrazine. In a control series the cells were exposed to saline alone. Samples were taken after 5, 15, 30, 60, and 90 minutes of incubation and examined for their 50 per cent lysis time with R1.

Fig. 3 shows that no decay occurs in the suspension of EAC'1 cells in saline (Fig. 3, curve I). It is also evident that 0.016 M hydrazine (an amount four times as great as that present in a dilution of 1:10 of R4) is absolutely indifferent with respect to the reactivity of the EAC'1 cells exposed to it (Fig. 3, curve II). A direct action of the hydrazine present in R4 upon the reactivity of EAC'1 can, therefore, be ruled out.

Samples of an EA suspension (8 amboceptor units) were mixed each with equal parts of a dilution of 1:10 of the following reagents prepared freshly from the same batch of complement: (*a*) Complement treated with ether; (*b*) Complement treated first with ether and then in a second step with hydrazine; (*c*) Complement treated with hydrazine; (*d*) Complement treated first with hydrazine and then with ether. The mixtures were incubated at 37°. The reactivity of the cells was analyzed at intervals during the incubation.

Fig. 3 (curves III to VI) summarizes the results. The controls indicate in accordance with previous experiments that in the mixture EA plus R4 the complex EAC'1 was formed quickly and decayed rapidly (curve VI) whereas in the mixture EA plus ether-treated complement no decay was detectable (curve III). Curve IV shows that hydrazine treatment applied to a complement previously treated with ether did not produce any change in the interaction with EA. On the other hand, curve V makes it evident that the decay-inducing power of R4 was markedly diminished, though not abolished, when the latter was treated with ether after having been treated with hydrazine. The outcome of this experiment cannot be explained satisfactorily by simply assuming that the decay-inducing property of R4 is an artifact acquired during the treatment with hydrazine. It seems more reasonable to assume that normal guinea pig serum itself possesses the power to inactivate cell-fixed C'1. To verify this hypothesis the influence of a decomplemented guinea pig serum on cell-fixed C'1 was studied.

An EAC'1 was prepared by exposing an EA (8 units of amboceptor) to a dilution of 1:60 of C'-ether at 3°. The cells were then washed and readjusted to 0.5×10^9/ml. Samples of this EAC'1 preparation were mixed with dilutions of 1:10 and 1:80 of decomplemented guinea pig serum and with samples of a decomplemented guinea pig serum which had been treated additionally with ether. The mixtures were incubated at 37° and at 3° respectively. The reactivity of the cells with respect to R1 was analyzed during the incubation.

Fig. 4 indicates that a serum which had been deprived of all of its C'1 and C'4 by treatment with an antigen-antibody complex possessed a considerable inactivating power upon EAC'1; the decay was more rapid at 37° (curve I),

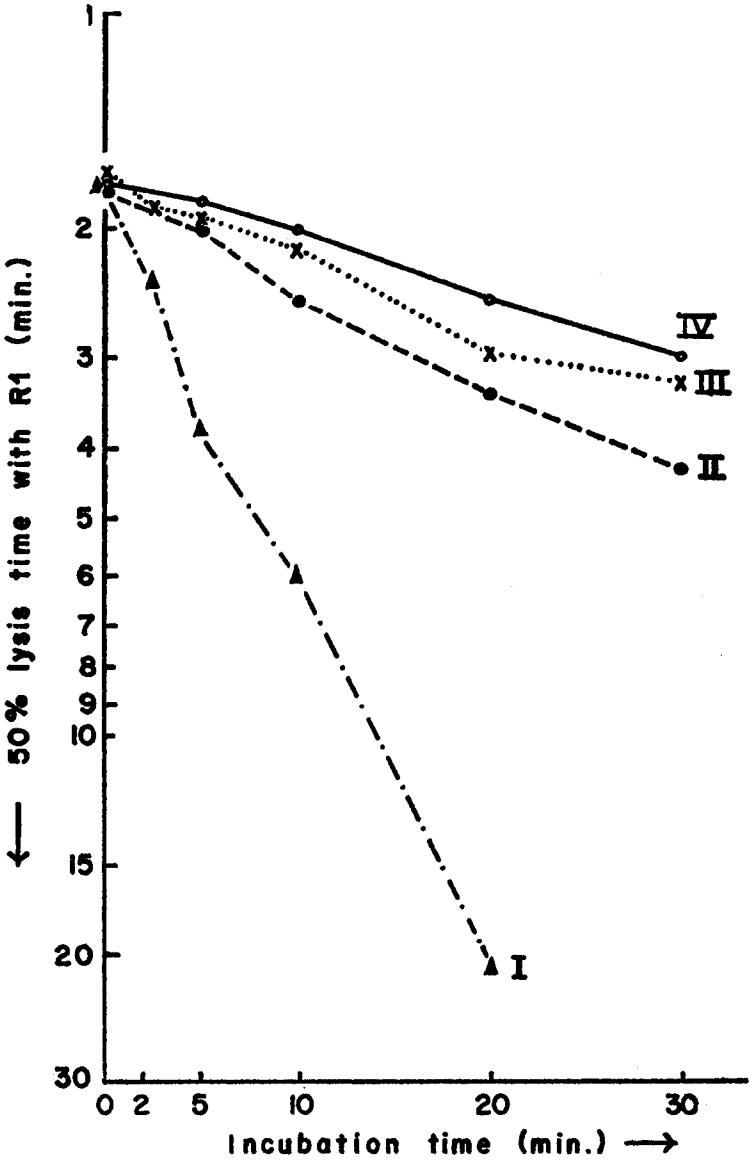

FIG. 4. Reactivity to R1 of an EAC'1 during its exposure to varying reagents. I, Exposed at 37° to decomplemented guinea pig serum 1:10. II, Exposed at 37° to decomplemented guinea pig serum 1:80. III, Exposed at 37° to a decomplemented guinea pig serum 1:10 treated additionally with ether. IV, Exposed at 3° to decomplemented guinea pig serum 1:10.

and slower at 3° (curve IV). It is noteworthy that ether treatment of the decomplemented guinea pig serum decreased its inactivating capacity sharply (curve III) and that the inactivation of EAC'1 proceeded more slowly when higher dilutions of decomplemented guinea pig serum were used (curve II). The inactivating capacity of decomplemented serum, therefore, shows the same characteristics as the inactivating capacity of R4, namely, a dependence upon temperature and reagent treatment with ether. These findings rule out the possibility that the inactivating power might be acquired by R4 during its treatment with hydrazine. Instead, they support the view that guinea pig serum itself, in the absence of the hemolytically active complement components, has the property to interfere with the capacity of cell-bound C'1 to react with C'4 and C'2.

The influence of the reaction volume on the interaction of EA and R4 was investigated. Two reaction mixtures were made using the same total amount of cells (2×10^9 EA) and of R4 (0.05 ml. with reference to the undiluted reagent). The first mixture had a final reaction volume of 2 ml. and the second a reaction volume of 8 ml. The mixtures were incubated at 37° and the reactivity of the cells to R1 was examined at intervals during the incubation.

Fig. 5 summarizes the results. It can be seen that a high reactivity was acquired by the cells in both mixtures. The decay of this reactivity progressed more slowly when the interaction took place in a large reaction volume (curve I). Yet in a smaller volume (¼ of the original) a more rapid decay was exhibited (curve II).

Experiments were undertaken to see whether the findings described above were valid also for cells sensitized with a reduced amount of amboceptor.

(*a*) Samples of cells sensitized with 2 amboceptor units (½ of the optimal titration dose) were exposed at 3° to a dilution of 1:10 of R4 and of C'-ether. The reactivity of the cells with respect to R1 was tested during the incubation.

Table II shows that in the system EA plus C'-ether the cell reactivity increased during the whole incubation period, yielding after 90 minutes a highly reactive EAC'1. The reactivity of the cells exposed to R4 on the other hand was feeble throughout the incubation period.

(*b*) The same experiment as described under paragraph (*a*) of this section was performed at an incubation temperature of 37°. In summary, the cells exposed to C'-ether under these conditions showed a moderately feeble though constant reactivity to R1 throughout the incubation period. The cells exposed to R4 showed a reactivity which was extremely feeble and which, moreover, disappeared completely after 5 minutes of incubation.

(*c*) An experiment was set up to study the stability of an EAC'1 that had been prepared in the cold by exposing suboptimally sensitized cells to C'-ether. When this complex was incubated in saline at 37°, no decay of its reactivity could be detected within an observation period of 90 minutes.

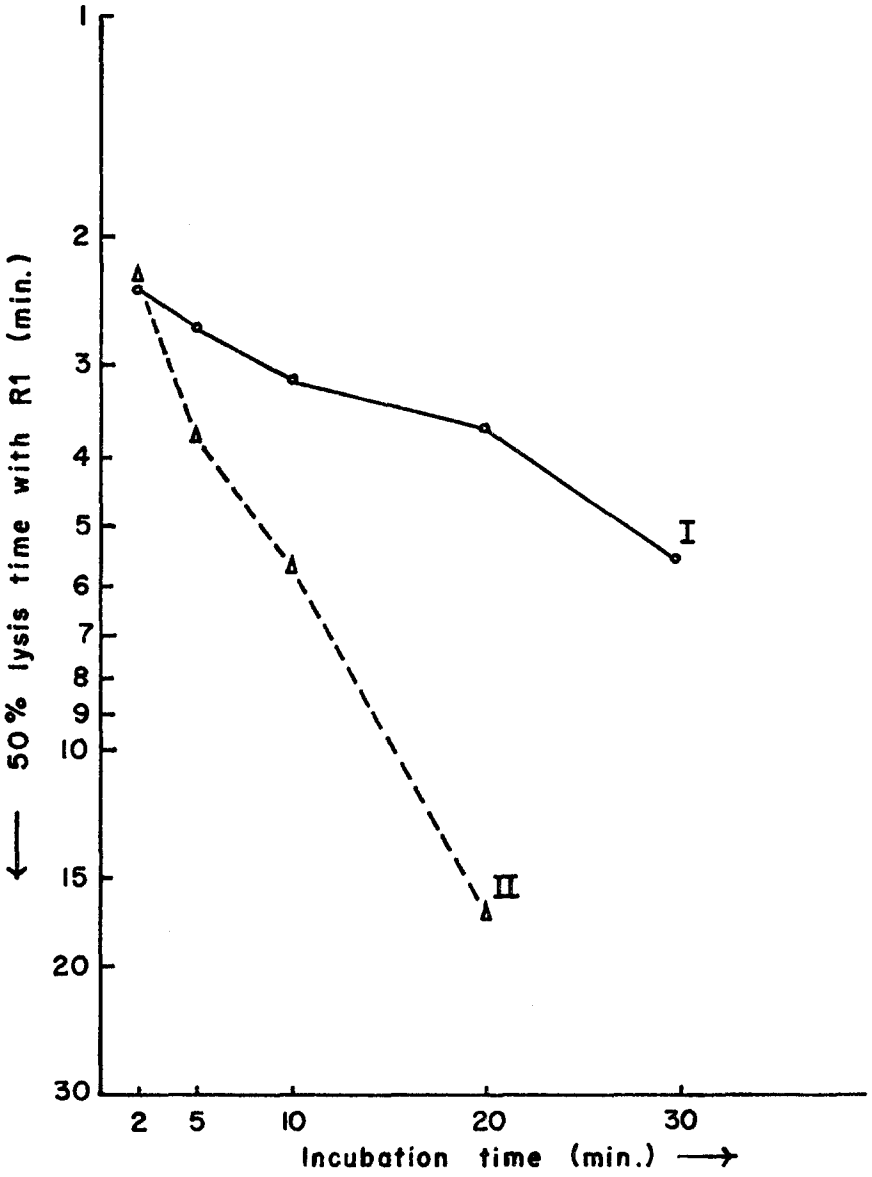

FIG. 5. Cell reactivity to R1 during the incubation of EA plus R4 in a large reaction volume (I) and in a small reaction volume (II).

It can be concluded, therefore, that with heavily sensitized cells an active EAC'1 can be obtained, even by using R4 (provided a short incubation time and low temperature). In using suboptimally sensitized cells, however, a satisfactorily active preparation of EAC'1 can be obtained only by interacting the cells with C'-ether in the cold for more than 1 hour.

TABLE II

Reactivity of Suboptimally Sensitized Cells to R1 during Their Exposure to R4 and to C'-Ether in the Cold

Incubation time of the reaction mixture	Reaction mixture I: EA (2 amboceptor units) plus R4		Reaction mixture II: EA (2 amboceptor units) plus C'-ether	
	50 per cent lysis time with R1	Lysis after 30 min. exposure to R1	50 per cent lysis time with R1	Lysis after 30 min. exposure to R1
min.	*min.*	*per cent*	*min.*	*per cent*
5	5.5	90	3.8	100
10	5.5	90	3.4	100
20	5.5	90	3.25	100
45	5.5	90	2.50	100
90	6.5	80	2.25	100

DISCUSSION

The findings presented in this paper explain some of the difficulties encountered in the preparation of the complex EAC'1 by exposing EA to R4. These difficulties are due to the fact that in the interaction of R4 and EA two processes take place: the fixation of C'1 to the cell, on the one hand, and the progressive inactivation of the fixed C'1, on the other. This inactivation is not spontaneous but is induced by an inactivation factor which is present not only in R4 but also in guinea pig serum decomplemented by treatment with an antigen-antibody complex. A subsequent paper will demonstrate that a similar inactivation factor can also be found in heat-inactivated guinea pig serum (9). The potency of the inactivating factor found in R4 and decomplemented serum can be markedly diminished by treatment with ether. A similar treatment of fresh guinea pig serum removes C'4 completely and leaves C'1 intact. In this preparation no trace of the inactivating capacity with respect to EAC'1 can be found. The inactivation of EAC'1 is diminished by low temperature and by increasing the reaction volume.

The finding that the complex EAC'1 is stable at 37° when suspended in buffered saline is in accordance with Laporte's observation. Mutsaars (10) reported that the reactivity of the complex EAC'1,4 with respect to endpiece even resists heating at 56°. On the other hand Levine and Mayer (1) found that in the presence of calcium and absence of magnesium ions the interaction of EA and complement at 34° results in the formation of the complex EAC'1,4,

the reactivity of which after reaching a peak decreases rapidly. On the basis of the data presented by these authors no answer can be given to the question as to which of the two fixed components decays, since the reactivity of the complex EAC'1,4 is evaluated by its hemolysis with a reagent containing only C'2 and C'3. The same question is also left open in the statement of Levine and Mayer (1) that the complex EAC'1,4 decays spontaneously at 37° when suspended in saline containing bovine albumin.

It is noteworthy that in an R4 the C'1 activity, as measured by titration, undergoes no reduction when the reagent is incubated at 37° in the absence of cells. Apparently the factor which inactivates fixed C'1 is indifferent with respect to free, non-fixed C'1. This, however, is not as surprising as it seems. Several observations suggest strongly that the properties of fixed C'1 are in many respects different from those of non-fixed C'1. Not only the above quoted observation of Mutsaars (10) but also those of Becker (8) regarding complement inhibition by DFP (Diisopropyl-fluorophosphate) demonstrate that fixed C'1 is susceptible to damages which do not affect the non-fixed form. Mayer and coworkers (11) showed that the reactivity of the complex EAC'1,4,2 to C'3 decays spontaneously at 37°. They concluded that the interaction of EAC'1,4,2 with C'3 involves a competition between the spontaneous decay of the cell reactivity to C'3 on the one hand and the fixation of C'3, on the other. The findings presented in this paper suggest that in addition to the decay of EAC'1,4,2, the interaction of EA and lytic complement components may be antagonized at an earlier step by a factor which by inactivating EAC'1 "competes" with the fixation of C'4 and C'2.

In studying the interaction of human complement and immune precipitates Lepow and Pillemer (12) concluded that the fixation of C'1 precedes all the other steps. The observations presented in this paper suggest, in accordance with Laporte's findings (4), that the very first step in the interaction of guinea pig complement with sensitized cells consists in the fixation of C'1, thus leading to the formation of EAC'1 which in turn reacts with C'4 and is converted thus into EAC'1,4. In experiments designed to study the interaction of EAC'1 with heat-inactivated and chelated complement, which is published in an associated paper (9), it was found, indeed, that EAC'1 is able to react with the component C'4 and is thus converted into EAC'1,4, whereas no reaction occurs between EA and C'4.

SUMMARY

The interaction of sensitized sheep erythrocytes (EA) with guinea pig complement inactivated by treatment with hydrazine or ether has been studied. In both cases EA is converted into EAC'1. The hemolytic reactivity of this complex with respect to a complement reagent that contains C'4, C'2, and C'3 (R1) decays rapidly in the presence of hydrazine-treated complement (R4) or

decomplemented guinea pig serum, but not in the presence of ether-treated guinea pig serum or of saline. The rate of decay of EAC'1 is dependent on the temperature, the reaction volume, and the amount of the inactivating reagent. The capacity of R4 and of decomplemented serum to inactivate EAC'1 can be markedly reduced by treatment with ether. The essential conditions for obtaining an optimally reactive EAC'1 consist in exposing EA to ether-treated guinea pig serum in the cold. This procedure yields a highly reactive EAC'1, even when the cells are sensitized with suboptimal amounts of amboceptor.

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