BIKEN JOURNAL Vol. 11, 101-110, 1968

TERMINAL STEP OF IMMUNE HEMOLYSIS

I. INHIBITION OF E* TRANSFORMATION BY URANYL ION

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(Received March 25, 1968)

SUMMARY Uranyl ion inhibits the step of E* generation. The reaction step which is blocked by uranyl ion is the step between activated E* and damaged E*. Kinetic analysis using both hemolytic and isotopic techniques revealed that cell membranes of uranyl ion blocked E* have small lesions through which low molecular weight materials, such as Rb ion, can escape while hemoglobin can not.

INTRODUCTION

Mayer and his colleagues showed that immune hemolysis is due to the action of 9 components of complement on the red cell membrane and that damage at a single site is sufficient to cause cell lysis. The mechanism of cell lysis has been postulated by GREEN *et al.* (1959) to be that low molecular materials leak out of the cells through small holes in the cell membrane and the cells become swollen and burst. Kinetic analysis of the terminal step of immune hemolysis by FRANK *et al.* (1965) revealed three successive steps in the process of cell damage, where 9 components reacted. They termed these intermediates E* precursor and activated E* which could be blocked by the presence of 0.09 M EDTA. Formation of damaged E*, a final intermediate in immune hemolysis, can be blocked by 25% bovine plasma albumin or 4 mM dextran 40 (SEARS *et al.*, 1964).

During a study of ATP dependent immune lysis of human red cells (MIYAMA *et al.*, 1965), it was found that formation of at least one E* intermediate was completely inhibited and cells failed to lyze in the presence of a low concentration of uranyl ion. We studied this step and the state of the cell surface of uranyl ion blocked E*, and determined the reaction sequence of E* generation which was blocked by uranyl ion.

MATERIALS AND METHODS

1. *Diluent*

Isotonic veronal buffered saline containing 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$ (VBS) was prepared as described by MAYER (1961). Isotonic veronal buffered sucrose solution (VB-sucrose) was prepared like VBS but contained 9.25% (w/v) sucrose instead of NaCl. A mixture of equal volumes of VBS and VB-sucrose (VBSS) was used throughout. VBS containing 0.01 M ethylene-diamine tetraacetate (0.01 M EDTA-VBS) was prepared by mixing 1 part of 0.1 M trisodium EDTA (pH 7.4) with 9 parts of VBS. Isotonic EDTA solution containing 0.1% gelatin (0.09 M EDTA) was prepared as described by FRANK *et al.* (1964). Isotonic buffer containing uranyl ion (U-VBSS, pH 7.4) contained 1 part of

0.01 M $UO_2(NO_3)_2$ or UO_2Cl_2 and 100 parts of VBSS. Isotonic buffer containing inosine, adenine, and glucose (IAG) was prepared as described by MIYAMA *et al.* (1965) except that it contained 0.0001 M RbCl instead of K_2HPO_4.

2. *Erythrocytes (E), rabbit hemolysin (A) and guinea pig serum (C′)*

Sheep blood, drawn aseptically, was preserved at 2°–4°C in the presence of 0.01 M EDTA. The erythrocytes used for isotopic experiments were obtained as described by MAYER (1961).

Rabbit hemolysin was purchased from Toshiba Chemicals Corp. and kept in a deep freezer.

The serum from pooled guinea pig blood was put into 2 ml ampules and lyophilized. Prior to the use, lyophilized serum was reconstituted by addition of the original volume of distilled water.

The standard method of titration of guinea pig complement of MAYER (1961) was slightly modified and the quantities of components of the reaction mixture were scaled down by a factor of 2.5.

3. *Sensitized erythrocytes (EA)*

Unless otherwise mentioned, EA was prepared by the method of INOUE *et al.* (1967).

4. *Components of guinea pig complement*

Partially purified C′2 was isolated by the method of BORSOS *et al.* (1961). Components C′3, C′5, C′6, C′7, C′8 and C′9 and reagent containing C′4, C′2, C′3, C′5, C′6 and C′7 (4–7 reagent) were obtained from Dr. K. Yonemasu of the Department of Bacteriology, Osaka University Medical School.

5. *Intermediate cells of immune hemolysis*

EAC′1,4 cells were prepared as described by INOUE and NELSON (1965, 1966). Two different methods were used for preparation of EAC′ 1,4,2,3,–5,6,7,8 cells (8-cells). In the first of these methods, 4–7 reagent was used as described by INOUE *et al.* (1967). A second method for preparing 8-cells was employed for isotope experiments: EAC′1,4,2 was allowed to react successively with C′3, C′5, C′6, C′7, and C′8, to give the corresponding intermediate cells.

6. *Radioactive RbCl*

^{86}RbCl was purchased from Daiichi Pure Chemicals Co. Ltd. The radioactivity of ^{86}Rb was measured with a conventional end-window Geiger-Müller counter (Kobe Industries Corp.).

RESULTS

1. *Inhibition of immune hemolysis by various metal ions*

The previous report (MIYAMA *et al.*, 1965) demonstrated that fresh human erythrocytes became spherical in shape on treatment with sodium fluoride, and their susceptibility to immune hemolysis increased to that of aged erythrocytes. We found that on treatment of fresh human erythrocytes with uranyl ion they also became spherical and concomitantly their ATP content decreased. However, when the cells were washed twice with VBSS after treatment with uranyl ion (0.001 M) and then allowed to react with antibody and complement, the degree of their lysis was less than that of fresh cells. After complete removal of uranyl ion by either thorough washing (more than 5 times) with VBSS or EDTA-VBS, the cells became susceptible to immune hemolysis like NaF-treated erythrocytes.

Thus it seemed that uranyl ion at relatively low concentration might inhibit some of the reaction steps of cell lysis in the immune hemolytic system. In the present work, the inhibitory effect of uranyl ion and its specificity in the immune hemolytic reaction were tested and compared with the other metal ions.

EA (0.5 ml, 4×10^8 cells/ml in VBSS) was added to solutions containing 1.5 ml of various dilutions of metal ions and 1.0 ml of 1/500 C′. After 60 min at 37°C, the tubes were centrifuged in the cold and the optical densities of the supernatants were measured at 541 mμ.

Table 1 shows the inhibition of hemolysis caused by various metal ions. Both UO_2Cl_2 and $UO_2(NO_3)_2$ effectively inhibited the lysis of EA at relatively lower concentrations than the effective concentrations of other metal ions tested. $FeSO_4$ which is an inhibitor of human C′9 (HADDING, 1967) inhibited the lysis of EA slightly at a concentration of 1×10^{-5} M but not 5×10^{-6} M. When more than 2 C′H50 of C′ were used in the presence of uranyl ion, partial lysis of cells occurred. The unlyzed

TABLE 1 *Inhibition of Immune Hemolysis by Metal Ions*

Metal ion tested	Final concentration			
	1×10^{-5} M	5×10^{-6} M	2.5×10^{-6} M	1.25×10^{-6} M
$ZnCl_2$	0.183	0.693	0.846	0.817
$CuSO_4$	0.109	0.529	0.625	0.660
$FeSO_4$	0.250	0.703	0.722	0.734
$UO_2(NO_3)_2$	0.000	0.085	0.470	0.595
UO_2Cl_2	0.000	0.075	0.481	0.585
Control		0.732		

The numbers represent the degree of lysis.

cells proceeded to lyze on removal of both complement and uranyl ion.

Ten ml of EA, at a concentration of 4×10^8 cells/ml in VBSS, were added to a mixture of 10 ml of 1/50 C′ and 40 ml of U-VBSS. After 15 min at 37°C, the mixture was poured into 60 ml of ice-cold U-VBSS and centrifuged in the cold. The cells were washed twice with ice-cold U-VBSS and resuspended in the same buffer at a concentration of 5×10^9 cells/ml. Flask A contained 10 ml of VBSS, and flasks B and C each contained 10 ml of U-VBSS. These flasks were placed in a water bath at 30°C. At zero time, each flask received 0.1 ml of the cell suspension and cells were kept in uniform suspension by constant shaking. At intervals, 1.0 ml aliquots were transfered from each flask to 6.5 ml of ice-cold U-VBSS. The tubes were centrifuged and the optical densities of the supernatants were measured at 413 mμ. After 75 min incubation, flasks A and B each received 1.4 ml of 0.1 M EDTA and flask C received 1.4 ml VBS. Again 1.0 ml aliquots were taken at intervals for 60 min.

Fig. 1 shows that when cells treated with C′ in the presence of uranyl ion were transferred to VBSS, lysis occurred without a lag period. Added EDTA removed the inhibition of the uranyl ion, presumably by chelation.

These findings suggest that the mechanism of inhibition by uranyl ion is different from those by other metal ions and the uranyl ion may specifically block the terminal step of

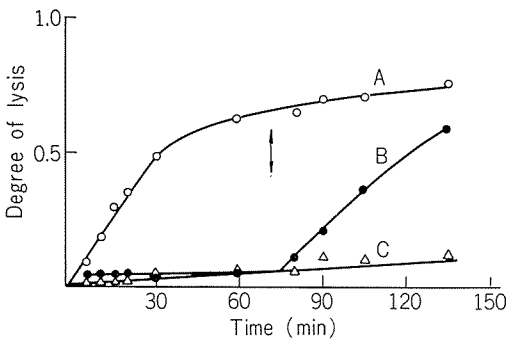

FIGURE 1 Lysis of uranyl ion blocked-E*.
Curve A: in VBSS.
Curve B and C: in U-VBSS.
At 75 min (indicated by arrow), EDTA was added to A and B.

immune hemolysis which does not require the action of components of complement.

2. *Generation of E* from EAC′ 1,4,2,3,5,6,7,8 (8-cells) and C′9 in the presence of uranyl ion*

Aliquots of 10 ml of 8-cells containing 1.5×10^8 cells/ml in VBSS were placed in three flasks A, B, and C, immersed in a water bath at 30°C. At zero time, flask A received 10 ml of C′9 in VBSS and flask B received 10 ml of C′9 in U-VBSS. Ten ml of VBSS were added to the control flask C. The flasks were shaken continuously and 1.0 ml aliquots were transferred at intervals to 6.5 ml ice-cold U-VBSS. The mixtures were centrifuged in the cold and the optical densities of the supernatants (Sup 1) were measured at 413 mμ. The cells were washed once with 10 ml of U-VBSS and resuspended in 7.5 ml of 0.01 M EDTA-VBS, and reincubated at 37°C for 90 min. These mixtures were centrifuged and the optical densities of the supernatants (Sup 2) were measured at 413 mμ. Samples were taken after 60 min, and EDTA at a final concentration of 0.01 M was added to flasks A and B, and 1.0 ml aliquots were taken in the same way as before. The ratios of lysis in Sup. 1 and Sup. 2 to the total lysis in the control were calculated as y1 and y2, respectively. y1 is the proportion of cells lyzed in time t, and y1+y2 represents the total E* formed in this time.

As shown in Fig. 2, 8-cells are not lyzed by C′9 in the presence of uranyl ion, and E* for-

mation from 8-cells with a limiting amount of C'9 is lower than that of the control: the E* formed within 60 min was about half of that formed in the absence of uranyl ion. This indicates that uranyl ion also inhibits the reaction between 8-cells and C'9. However, as shown in Fig. 3, when a large excess of C'9 was used, the extent of E* formation was the same as that without uranyl ion and the addition of EDTA completely restored cell lysis. These experiments suggested that a step(s) in the reaction sequence of E* transformation might be specifically blocked by uranly ion.

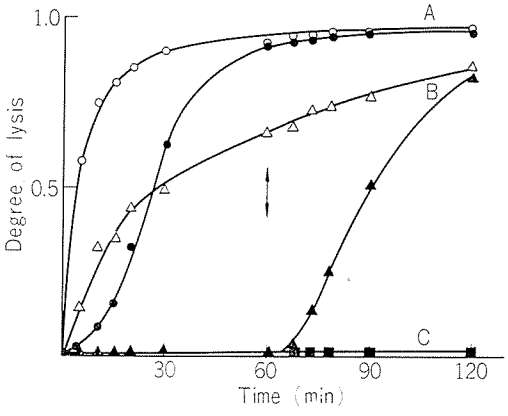

FIGURE 2 E* generation from 8-cells and C'9 (1/1000) in the presence and absence of uranyl ion.
Curve A: in the absence of uranyl ion.
Curve B and C: in the presence of uranyl ion.
○——○ and △——△: E* formation.
●——●, ▲——▲ and ■——■: cell lysis.
At 60 min, EDTA was added to A and B.

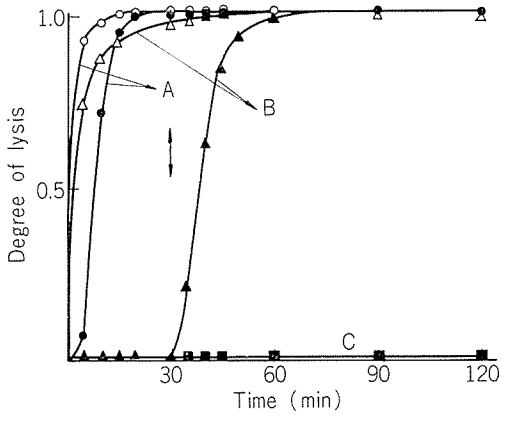

FIGURE 3 E* generation from 8-cells and C'9 (1/100).
The notations are the same as for Fig. 2.
At 30 min, EDTA was added to A and B.

3. *Effect of uranyl ion concentration*

Ten ml of 8-cells at a concentration of 1.5×10^8 cells/ml in VBSS were incubated with 10 ml of C'9 (1/100) in U-VBSS at 37°C for 30 min. The unlyzed cells were washed twice with U-VBSS and resuspended in ice-cold VBSS at a concentration of 5×10^8 cells/ml. Immediately 0.1 ml of the suspension was added to 5.0 ml volumes of various concentrations of metal ions. After 60 min at 30°C, the tubes were centrifuged and the optical densities of the supernatants were read at 413 mμ.

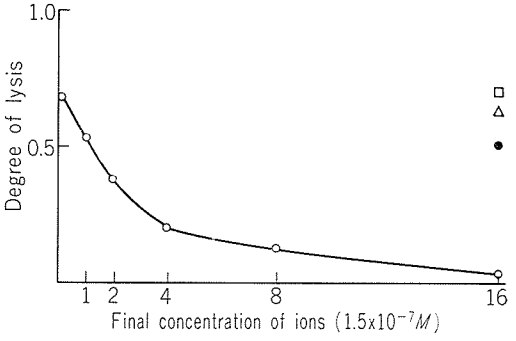

FIGURE 4 Effect of concentration of uranyl ion.
○: UO_2Cl_2 ●: $ZnCl_2$
△: $CuSO_4$ □: $FeSO_4$

As shown in Fig. 4, inhibition depended upon the concentration of uranyl ion and other ions tested did not cause inhibition, even at the highest concentrations tested.

4. *The stability of E* blocked with uranyl ion*

FRANK *et al.* (1965) demonstrated that when E* is blocked by 0.09 M EDTA it is relatively stable and the S* sites are not lost in EDTA medium. MIYAMA *et al.* (1968) showed that the lysis of human E* depends upon their ATP

contents: S* sites are lost during the synthesis of ATP. The following experiment was on the possible decay of uranyl ion blocked E*s prepared from fresh (ATP-rich) and aged sheep red cells.

A mixture of 10 ml of 8-cells and 10 ml of C'9 (1/100) in U-VBSS was incubated at 30°C for 30 min. The cells were thoroughly washed and resuspended in U-VBSS at a concentration of 1.5×10^9 cells/ml. One ml of a suspension of uranyl ion-blocked E* was pippeted into a flask containing 19 ml of U-VBSS, immersed in a water bath at 30°C. At

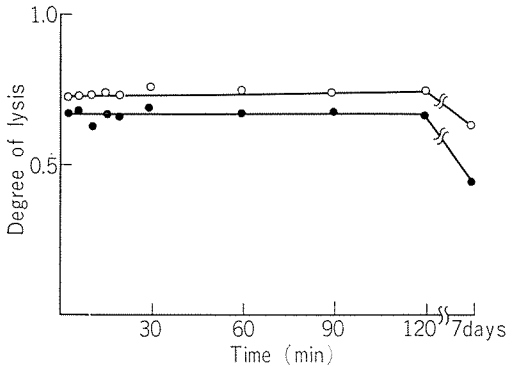

FIGURE 5 Stability of E* blocked by uranyl ion.
○——○: E* from aged cells.
●——●: E* from fresh cells.

intervals, 1.0 ml aliquots were transferred from the reaction mixture to 1.5 ml of 0.01 M EDTA-VBS, and then incubated at 37°C for 90 min. After incubation, each tube received 5.0 ml of ice-cold saline and was centrifuged.

As shown in Fig. 5, there was no decay of S* sites of cells prepared either from fresh or aged red cells.

5. *Analysis of terminal step blocked by uranyl ion*

FRANK *et al.* (1965) reported that 0.09 M EDTA inhibits the early step of E* transformation but not the final step, which is prevented by either 25% bovine plasma albumin or 4 mM dextran 40 (SEARS *et al.*, 1964). The following experiment was to see which step the uranyl ion blocks.

A suspension of 8-cells at a concentration of 1.5×10^8 cells/ml in VBSS was mixed with an equal volume of 1/100 C'9 in U-VBSS and incubated at 30°C for 30 min. The cells were washed twice with ice-cold U-VBSS and resuspended at a concentration of 1.5×10^9 cells/ml in U-VBSS. To flasks A, B, and C, respectively, were added 10 ml of 0.09 M EDTA, U-VBSS and 4 mM dextran 40. At zero time, 0.5 ml of a suspension of uranyl ion-blocked E* was added to all the flasks and they were incubated at 30°C with constant shaking. At intervals, 1.0 ml aliquots were taken into 6.5 ml of ice-cold VBSS. The tubes were immediately centrifuged and the optical densities of supernatants were measured at 413 mμ.

Fig. 6 shows that when uranyl ion-blocked E* was transferred to 0.09 M EDTA medium the cells started to lyze without any lag period, whereas no lysis was observed in the U-VBSS medium. On the other hand, when transferred into 4 mM dextran 40, the cells remained intact for the first 30 min and then gradually lyzed, probably due to instability of E* in 4 mM dextran 40.

To find which steps were blocked by uranyl

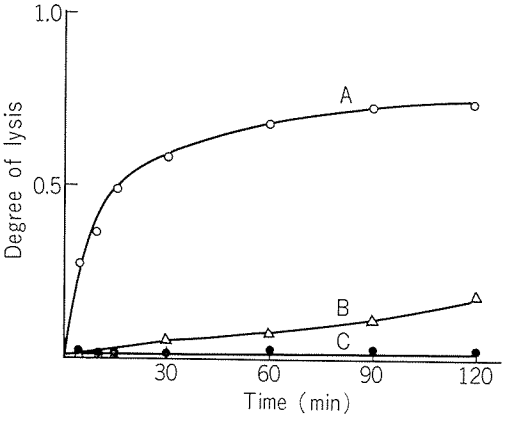

FIGURE 6 Lysis of E* blocked by uranyl ion in 0.09 M EDTA, 4 mM dextran, and U-VBSS.
Curve A: 0.09 M EDTA.
Curve B: 4 mM dextran 40.
Curve C: U-VBSS.

ion, it was necessary to see whether E* prepared in dextran 40 could be protected in medium containing uranyl ion.

E* blocked by dextran 40 was prepared as follows: 8.0 ml of EA at a concentration of 1×10^8 cells/ml in 4 mM dextran 40 were incubated with 4 ml of C′ (1/100) in the same medium at 30°C for 30 min. The cells were centrifuged and washed once with a large amount of 4 mM dextran 40, and then resuspended in the same medium at a concentration of 5×10^8 cells/ml. About 40% of EA were lyzed during the centrifugation and washing. E* blocked by uranyl ion was also prepared from EA by the method mentioned in Paragraph 1 and resuspended in U-VBSS at a concentration of 5×10^8 cells/ml. To tubes containing 5 ml of 0.01 M EDTA-VBS, U-VBSS, and 4 mM dextran 40, respectively, were added 0.1 ml of E* prepared in the presence of either dextran or uranyl ion.

As shown in Table 2, lysis of dextran-blocked E* was partially but not completely prevented by uranyl ion. On the other hand, uranyl ion-blocked E* did not lyze in either U-VBSS or 4 mM dextran 40. These results show clearly that uranyl ion can block the step between the 0.09 M EDTA-, and 4 mM dextran 40-steps.

GREEN *et al.* (1959) and SEARS *et al.* (1964) demonstrated that E* blocked by 25% albumin has small holes on its surface through which low molecular materials such as Na^+ and K^+ pass. FRANK *et al.* (1965) also suggested that activated E*, blocked by 0.09 M EDTA, has lesions on its surface. The following experiment was to determine the size of the lesions produced on the surface of E* blocked with uranyl ion.

TABLE 2 *Steps in E* Transformation*

	Extent of lysis in		
	0.01 M EDTA-VBS	U-VBSS	4 mM dextran
E* prepared in U-VBSS	0.660	0.045	0.105
E* prepared in 4 mM dextran 40	0.455	0.392	0.135

A suspension of sheep erythrocytes collected in Alsever's solution was washed 5 times with VBS and stored at 0°C for 3 days to reduce the intracellular content of potassium. After the cold storage of the E suspension, an intermediate, EAC′1,4,2 was prepared. The cells were resuspended at a concentration of 1×10^9 cells/ml in 20 ml of IAG containing 500 μC of ^{86}RbCl and incubated at 37°C for 2 hr (HASHIMOTO *et al.*, 1963). During the incubation, an intermediate, EAC′1,4,2 was converted to EAC′1,4 and labeling with intracellular ^{86}Rb occurred. The cells were centrifuged and washed twice with VBSS. Ten ml of EAC′1,4 at a concentration of 1×10^9 cells/ml were mixed with 4 ml of C′2 and 8 ml of C′3. After incubation at 30°C for 10 min with constant shaking, the cells were washed twice and resuspended in ice-cold VBSS at a concentration of 1×10^9 cells/ml. Ten ml of EAC′1,4,2,3 were allowed to react successively with the following reagents: 4 ml of C′5 and C′6, 4 ml of C′7, and 1.2 ml of C′8. Each step in the formation of intermediates was followed by washing the cells twice with VBSS before the next step. The resulting cells were the intermediate EAC′1,4,2, 3, 5, 6, 7, 8 (8-cells). Volumes of 6 ml of 8-cells at a concentration of 5×10^8 cells/ml in VBSS were put into three flasks A, B and C which were warmed in a water bath at 30°C. At zero time, flask A received 6 ml of 1/100 C′9 in VBSS and flask B received 6 ml of 1/100 C′9 in U-VBSS. Flask C, as control, received 6 ml of VBSS alone. At intervals, 1 ml aliquots were pippeted into 1.0 ml of ice-cold U-VBSS and the supernatants were separated by centrifugation. The supernatants were transferred to sample dishes, dried under an infrared lamp, and their radioactivity was counted with a Geiger-Müller tube. The total count of ^{86}Rb in the cells were estimated from the counts of a suspension of 1 ml of reactant with 1 ml of distilled water. The ratio of the release of ^{86}Rb to the total ^{86}Rb content was calculated as shown in Fig. 7. The release of ^{86}Rb from control 8-cells in flask C was about 20% at 60 min. In parallel, 0.2 ml aliquots were transferred from each flask to 5 ml of U-VBSS and the mixtures were centrifuged in the cold. The supernatants (Sup 1) were removed and the cells were washed once with 10 ml of U-VBSS. The cells were resuspended in 5.0 ml of 0.01 M EDTA-VBS and reincubated at 37°C for 90 min with constant shaking. Then they were centrifuged and the supernatants (Sup 2) were removed. The optical densities

of Sup. 1 and Sup. 2 were measured at 413 mμ.

As shown in Fig. 7, the release of ^{86}Rb occurred within 2 min and the kinetic curve of the release of ^{86}Rb was superimposable on that of total E* formed. HINGSON *et al.* (1966) also reported that the release of ^{86}Rb started quickly in the reaction between EAC'1,4,2 and C'EDTA. In the presence of uranyl ion, however, the release of ^{86}Rb occurred immediately after the formation of E*, whereas no release of hemoglobin was seen.

These results confirmed that the action of uranyl ion is on E* transformation: uranyl ion inhibits the process from activated E* to damaged E* but does not block either the formation of small holes or the leakage of low molecular weight materials.

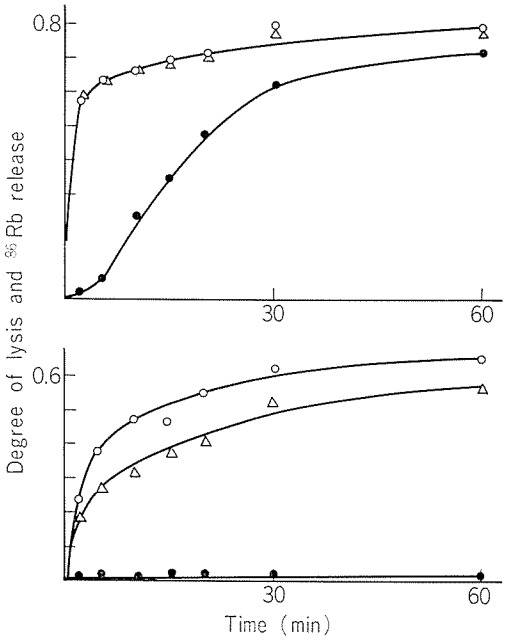

FIGURE 7 E* formation and ^{86}Rb release.
Upper: in the absence of uranyl ion.
Lower: in the presence of uranyl ion.
○——○: E* formation.
●——●: cell lysis.
△——△: release of ^{86}Rb.

DISCUSSION

In studies on ATP dependent lysis of human erythrocytes, uranyl ion was found to be a potent inhibitor of lysis of red cells. The lysis of EA with 1 C'H50 of C' was completely inhibited by uranyl ion at a concentration of 5×10^{-5} M. The formation of E* from EA in the presence of uranyl ion at the same concentration requires more than 3 C'H50 of C', suggesting that uranyl ion either inactivates C' or partially inhibits some steps in the intermediate formation. It was demonstrated that uranyl ion at higher concentration (0.001 M) denatures some components of the complement. However, a low concentration of uranyl ion (less than 5×10^{-5} M) failed to inhibit the generation of either intermediate of C'6, C'7, and C'8 steps. On the other hand, the IA reactivity of C'3 (2 C'IA50) was completely inhibited by uranyl ion at a final concentration of 5×10^{-5} M. The generation of intermediates of the C'1, C'2, C'5 and C'9 steps were partially inhibited by uranyl ion at a concentration of 5×10^{-5} M. The decreased amount of E* formation, as seen in Fig. 2, would be due to inhibition by uranyl ion of the generation between the C'8 and C'9 steps. Although uranyl ion inhibits some of the components of complement, it completely blocks the lysis of E* carring more than 2 S* sites (average number of mebrane lesions/cell) at a concentration as low as 5×10^{-6} M.

MCQUILLEN (1950) demonstrated that uranyl ion combines with phospholipid and modifies the charge of the bacterial surface. TACHIBANA *et al.* (1962) and TACHIBANA (1963) demonstrated that the shape of the red cell depends upon the reactivity of Mg-dependent ATPase located in cell membrane, and that the ATPase associated with phospholipid plays an important role in active transport of the cells. They also demonstrated that treatment with phospholipase A from snake venom destroyed active transport of the cell membrane. On the basis of these observations, it is speculated that uranyl ion modifies the phospholipid

moiety of the red cell surface and the resulting modification of the cell surface may be reversible because E* generation is restored by the addition of EDTA.

GREEN *et al.* (1959) reported that when cells were treated with C′ and antibody they released low molecular materials prior to cell lysis, and these workers suggested that the loss of small molecules created an osmotic imbalance between the inside and outside of the cells, which resulted in an influx of water into the cells. They showed that 25% bovine plasma albumin in the medium could maintain the osmotic pressure and prevent cell lysis. Although uranyl ion does not stabilize the osmotic pressure, it can prevent E* with small holes on its surface from lysis.

These results suggest that the process of transformation from activated E* to damaged E* may be controlled by a mechanism associated with membrane phospholipid, which can combine reversibly with uranyl ion to form an inert complex.

ACKNOWLEDGEMENTS

The authors wish to express their sincere appreciation to Drs. T. Amano and K. Inoue of the Department of Bacteriology, Osaka University Medical School, for their helpful suggestions and criticisms throughout this work. They also wish to thank Dr K. Yonemasu for his generous gifts of components of guinea pig complement.

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