

## MECHANISM OF COMPLEMENT-INDUCED CELL LYSIS DEMONSTRATION OF A THREE-STEP MECHANISM OF EAC1-8 CELL LYSIS BY C9 AND OF A NON-OSMOTIC SWELLING OF ERYTHROCYTES

GÜNTER VALET AND WOLFGANG OPFERKUCH

*From the Max-Planck-Institut für Biochemie, Department of Experimental Medicine, 8033 Martinsried and Lehrstuhl für Medizinische Mikrobiologie, Ruhr-Universität, 463 Bochum, Germany*

Sheep erythrocytes were sized by an electrical method during lysis by antibody and complement. No volume changes were observed up to the EAC1-8 cell intermediate. Three steps could be distinguished in the reaction of C9 with EAC1-8 cells: fixation of C9 to EAC1-8 cells, a process which proceeds also at 0°C; temperature-dependent swelling of the EAC1-9 cells; and lysis of the cells.

We could show that swelling of the erythrocytes induced by the complement reaction is independent of protein osmotic forces and is caused by an alteration of the erythrocyte membrane.

The mechanism of membrane damage due to the complement reaction is not well understood. Erythrocytes seem to disrupt suddenly under the action of antibody and complement (1) and typical lesions can be seen in the membrane of complement-lyzed erythrocytes by electron microscopy (2, 3). Although complement-dependent generation of lyso-lecithin has been thought to cause cell lysis (4), these findings have not been confirmed (3, 5-7). Alternatively, cell lysis could be due to a physicochemical interaction of the terminal complement components C5-C9 with the cell membrane since it has been observed with filipin or saponin (8, 9). Based on the present experimental evidence, the doughnut model of complement-mediated erythrocyte lysis has been developed (10).

As an early effect of the action of the C5-9 complex (11, 12) on the cell membrane the cell loses low molecular constituents such as K<sup>+</sup>, free amino acids, and sugars, and influx of Na<sup>+</sup> occurs (13, 14). According to Green *et al.* (13) this leads to cell swelling due to the osmotic pressure of the intracellular protein. The cell swells until the membrane is stretched to such an extent that cell protein is lost into the medium through "functional holes". Some of the Na<sup>+</sup> and K<sup>+</sup> exchange phenomenon might be associated with an impairment of the K<sup>+</sup>/Na<sup>+</sup> pump. This has been shown to occur during complement lysis of erythrocytes with genetically determined high and low intracellular K<sup>+</sup> content (15-17). The terminal stages of complement lysis can be inhibited by high concentrations of albumin and EDTA (13, 18, 19). Recently high intracellular concentrations of cyclic AMP have been found to inhibit complement-induced lysis of mast cells (20) and Moloney virus-transformed lymphocytes become resistant to complement lysis (21).

The purpose of this work was to investigate the mechanism of complement-mediated cell damage in more detail by study-

ing cell membrane alterations through measurements of erythrocyte volumes during lysis.

### MATERIALS AND METHODS

**Buffers and Substances.** Ethylenediaminoacetic acid buffer 0.09 M (EDTA),<sup>1</sup> pH 7.6, and low ionic strength sucrose Veronal-buffered saline with 0.1% gelatin (VBS-S) were prepared as described (18, 22). Glucose was used usually instead of sucrose with or without 0.1% gelatin (VBS-G) (23). For cell counting and volume distribution measurements the buffers were used without gelatin. Albumin solutions were made from stock solutions of twice concentrated VBS-G or 0.18 M EDTA, pH 7.6, and albumin to give a final 36% concentration of albumin in VBS-G or 0.09 M EDTA. Bovine serum albumin (BSA) was 100% electrophoretically pure from Behringwerke, Marburg, Germany and 1-10-orthophenanthroline was from Merck, Darmstadt, Germany.

**Complement components.** Human and guinea pig complement components C2-C9 were in functionally pure form from Cordis Co., Miami, Fla. Guinea pig C1 (C1gp) was prepared as described (24).

**Cell intermediates.** Sheep erythrocytes (E-cells) were obtained from Biologische Arbeitsgemeinschaft, Lich, Germany. They were incubated with rabbit-anti-sheep erythrocyte anti-serum (Behringwerke) at optimal concentration in VBS-S. EAC1, EAC14, EAC142, EAC1423; E<sup>x</sup> cell intermediates<sup>1</sup> were made according to published methods (22, 25, 18, 19). Cell intermediates were made with human or guinea pig complement components except for C1gp and oxidized C2hu (25). EAC1-3 cells were incubated in VBS-G for 60 min at 37°C with C5-C7 to give EAC1-7 cells. C8 in sublytic amounts was then attached to the cells by incubation in VBS-G for 15 min at 30°C. Finally EAC1-8 cells were lyzed with C9 in VBS-G at 30°C. Lysis was determined spectrophotometrically measuring the amount of hemoglobin released into the supernatant at 412 nm. In some experiments EA cells were lyzed with appropriate dilutions of human or guinea pig serum in VBS-G at 30°C or 37°C. The final cell concentration in all assays was 4 × 10<sup>7</sup> cells/ml.

**Cell counts and volume measurements.** Erythrocytes were

<sup>1</sup> Abbreviations used in this paper: Nomenclature of complement: Bull. W.H.O., 39: 935, 1968; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; C1gp, guinea pig C1; E<sup>x</sup>-cells, EAC1-9 cells; VBS, Veronal-buffered saline; VBS-S, VBS containing 5% sucrose; VBS-G, VBS containing 5% glucose; VBS-EDTA, VBS containing 0.02 M EDTA.

counted with a Coulter A particle counter with a 100  $\mu\text{m}$  orifice, amplification 2 in VBS-G or 0.09 M EDTA, pH 7.6, buffer with current and threshold values adjusted to impulse height.

The volume distribution curves of the erythrocytes were also measured by the electrical method. An orifice with 50- $\mu\text{m}$  diameter and approximately 40- $\mu\text{m}$  length was used. The orifice was modified by a particle beam attachment (26, 27). This modification has the advantage that all particles are sized under the same electrical and hydrodynamical conditions. Measurements can also be made with erythrocytes suspended in highly concentrated 36% albumin solutions. Furthermore, fewer cells are needed and contact of the cells with products of electrolysis was avoided. All measurements were performed at 25°C in VBS-G or 0.09 M EDTA. The aperture current was 0.35 mA. Between 800 and 1200 particles per second were measured at a suction of 0.1 kp/cm<sup>2</sup>. The impulses were amplified and registered according to their maximal height in a 64 class multi-channel analyzer (Telefunken, Germany). The resulting volume distribution curves were plotted, punched on tape, and analyzed by a computer for linear Gaussian normal distributions of volume by the method of least squares (28). Between 500 and 1000 iteration steps were made to get best fits for each curve. Three dimensional plots (28) were drawn from the analyzed curves to give a better impression of the changes of the volume distribution curves during lytic experiments.

Absolute volumes were calculated from calibrations of the apparatus with latex particles of known volume (29) (Particle Information Service, Los Altos, Calif.), erythrocytes whose mean volume (MCV) had been determined from hematocrit and cell concentration and by an electrical calibration method (29).

In some experiments the erythrocytes were photographed in the orifice (30) to determine their form under the conditions of the volume measurements. From these photographs the form factor (31, 32) was determined which is necessary for the calculation of absolute volumes of particles (29).

For the determination of the specific electrical resistance the erythrocytes were suspended in a 0.3 M sucrose solution to which increasing amounts of NaCl were added. The erythrocyte suspension was measured in the sizing apparatus with an

aperture current of 50  $\mu\text{A}$  and the polarity of the impulses was observed on an oscilloscope. If the particle resistance was higher than the resistance of the suspending medium the electrical impulses were positive, if it was approximately equal the impulses became small and an equal number of positive and negative impulses were observed, and if it was lower all impulses became negative. The specific electrical resistance of the sucrose/NaCl solution was determined in  $\Omega\text{cm}$  at 25°C when an equal number of positive and negative impulses was observed. This value was taken as the mean specific resistance of the erythrocytes in  $\Omega\text{cm}$ .

## RESULTS

**Methodology.** The apparent volume of a particle in the electrical sizing method is influenced by the absolute volume, by the shape, and by the electrical resistance of the particle during its way through the orifice. Erythrocytes were photographed in the orifice under the conditions used for the volume determination (Fig. 1) to get an impression how they are deformed. E and EAC4 cells behaved quite similarly. A mean form factor of  $1.27 \pm .03$  ( $\pm$  standard error,  $n = 50$ ) was determined from the photographs. Intact and water-lyzed erythrocytes had a specific electrical resistance of greater than 2 k $\Omega\text{cm}$  as compared to 115  $\Omega\text{cm}$  for VBS-G and 65  $\Omega\text{cm}$  for 0.09 M EDTA at 25°C. This resistance was 17 to 30 times greater than that of the suspending medium. Therefore absolute volumes of erythrocytes could be calculated (29). With the form factor of 1.27 a mean volume of  $28 \pm 1 \mu\text{m}^3$  ( $n = 5$ ) was determined for EA cells.

**Correlation between supernatant hemoglobin and electrical cell counts during complement lysis.** EAC1-8 cells were incubated at 30°C with C9. Lysis was followed by spectrophotometric determination of supernatant hemoglobin and electrical counting of the cells (Fig. 2). Although lysis proceeded as shown by the increasing hemoglobin concentration in the supernatant, the cell concentration remained constant, indicating that both, intact erythrocytes and ghosts, were measured by electrical counting and sizing.

**Volume distribution curves of E to EAC1-9 cell intermediates.** None of the intermediates up to EAC1-8 showed any



Figure 1. Sheep erythrocytes photographed on their way through the orifice. They were suspended in VBS-S and sucked from right to left through the orifice. The length of the orifice was approximately 100  $\mu\text{m}$ .

significant change of their mean volume and their volume distribution curves. The curves were best approximated by a single normal distribution of volume (Fig. 3A). When C9 was added to EAC1-8 intermediates at 30°C or 37°C volume distribution curves with two peaks were observed (Fig. 3B) which were best approximated by two normal distributions of volume. The population of small cells had the volume of EAC1-8 cells. The population of large cells was newly generated by the action of C9 (large and small cell population will be called simply large and small cells in subsequent paragraphs). Figure 4 depicts the occurrence of the population of large cells in the course of EAC1-8 cell lysis by addition of C9. The small cells decrease with time and the large cells appear after a short lag phase. The generation of large cells is C9 dependent since an increase of the C9 concentration resulted in a higher proportion of large cells (Fig. 5A, B).

Several functional stages, such as the precursor, activated, and damaged E cells, have been described during the final step of complement lysis (18, 19). We, therefore, investigated as to whether certain of these prelytic stages could be correlated with the observed volume changes. In addition we studied the substitution of C9 by phenantroline (33) in this reaction.

**EAC1-8 cells + C9 at 0°C.** When EAC1-8 cells were incubated with C9 at 0°C no lysis or swelling of the cells occurred (Fig. 6). Aliquots of cells taken from the reaction mixture at different time intervals were washed twice in cold buffer and then incubated at 30°C. Lysis occurred as shown by the dashed line (Fig. 6) indicating that EAC1-8 cells had fixed C9 at 0°C.

**EAC1-8 cells + C9 in 0.09 M EDTA buffer, pH 7.6.** EAC1-8 cells were incubated with C9 in VBS-G for 10 min. at 30°C. The cells were then washed twice at 0°C and resuspended in 0.09 M EDTA buffer, pH 7.6, at 30°C. No lysis was observed (Fig. 7) although formation of large cells proceeded. It was concluded, therefore, that EDTA inhibited lysis, not, however, swelling of the cells.

**Phenantroline as a C9 substitute.** When phenantroline was used as a C9 substitute the greatest amount of large cells was attained at a concentration of phenantroline of 5 mM.

**Effect of albumin on cell swelling.** EA cells were suspended

in VBS-G with or without 36% BSA added to counterbalance the osmotic pressure of intracellular hemoglobin. Then whole guinea pig serum was added and the mixture was incubated at 30°C. Lysis and volume distribution curves were measured at different time intervals (Table I). Lysis but not swelling of the

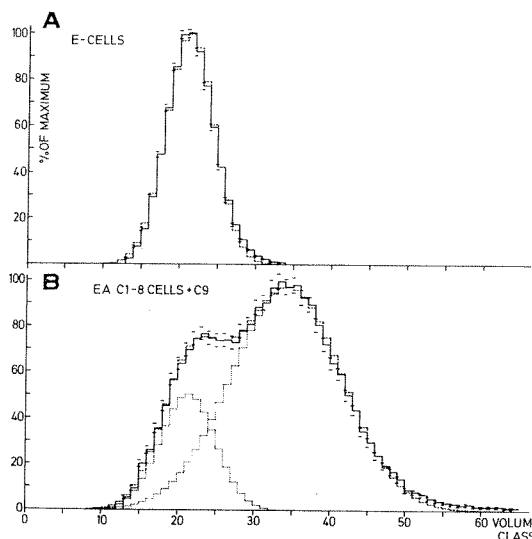


Figure 3A and B. Changes of the volume distribution curves of EAC1-8gp cells on addition of C9gp. A, the volume distribution curve of E-cells is shown. The curve of EAC1-8 cells is very similar. The experimental curve and the computer approximation by a linear normal distribution are drawn. The short horizontal bars are the  $\pm 2s$  deviations of the statistical counting error ( $s = \sqrt{n}$ ,  $s$  = standard deviation,  $n$  = number of erythrocytes/volume class, 8.4 and  $10.7 \times 10^4$  cells (A, B) were measured per volume distribution curve, respectively, and one volume class corresponds to  $1.40 \mu\text{m}^3$ ). The E- and EAC1-8 cell volume distributions were best approximated by one normal distribution of volume. B, two volume peaks are visible in the curve. The curve is best approximated by two normal distributions of volume, one with the volume of EAC1-8 cells (small cells) and one with a larger mean volume (large cells).

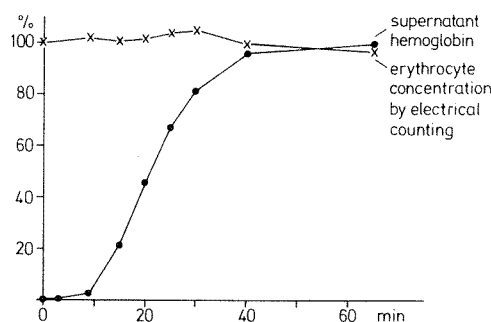


Figure 2. Incubation of EA cells with whole guinea pig complement at 37°C. Aliquots of the assay were taken at different times and analyzed for cell concentration with a Coulter counter and for hemoglobin in the supernatant with a spectrophotometer. Lysis proceeds as shown by the increasing hemoglobin in the supernatant. The cell concentration, however, remains unchanged. This means that erythrocyte ghosts are impermeable for the electrical current and that both erythrocytes and erythrocyte ghosts are measured by electrical counting and sizing.

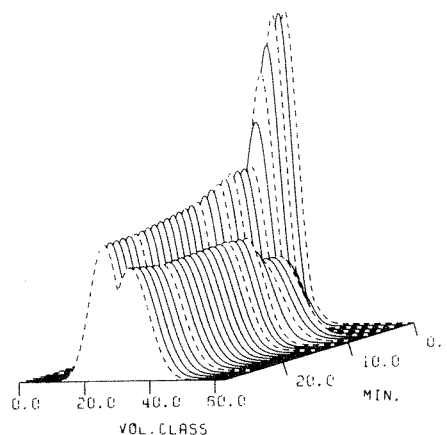


Figure 4. Changes of the volume distributions curve of EAC1-8hu cells on addition of C9hu at 30°C (2 CH50 C9). The volume distribution curves were measured at different times of the reaction. The dashed curves are experimental curves, the solid curves were calculated by linear interpolation. After a short incubation time large cells are formed, whose concentration remains constant throughout the experiment. One volume class corresponds to  $1.16 \mu\text{m}^3$ .

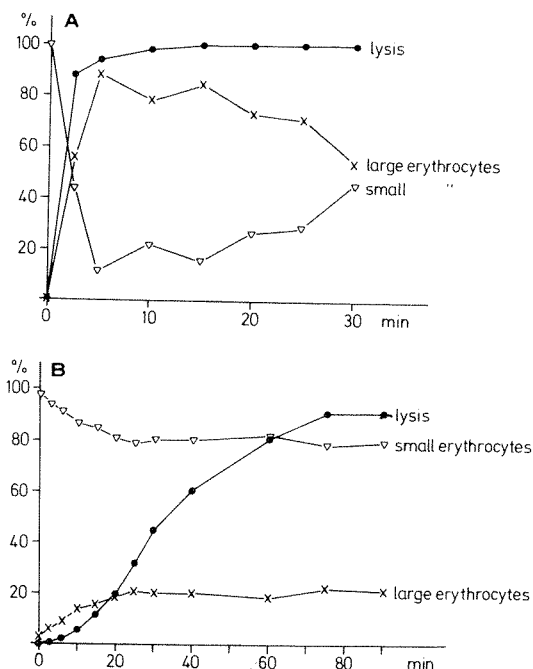


Figure 5A, B. Incubation of EAC1-8gp cells with C9gp at 30°C. The number of small and large cells was compared with the degree of lysis. In fast complement lysis (50 CH<sub>50</sub> C9) (upper panel) the concentration of large cells parallels the hemoglobin in the supernatant at the beginning of the experiment. In slow complement lysis (2.5 CH<sub>50</sub> C9) (lower panel) the concentration of large cells remains inferior to lysis.

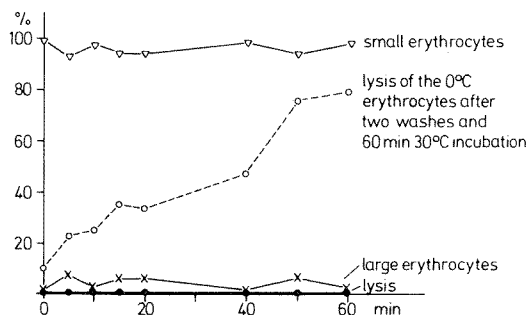


Figure 6. Incubation of EAC1-8hu cells with C9hu at 0°C. Aliquots of the assay were taken at different times and kept at 0°C. They were analyzed in the following way: first, the erythrocytes were sized electrically and the percentage of large and small erythrocytes was calculated; second, the degree of lysis was determined from the supernatant hemoglobin; third, one part of the cells was washed twice in the cold with VBS-G and then incubated at 37°C. Lysis occurred, showing that EAC1-8 cells had taken up C9 at 0°C without swelling.

erythrocytes was inhibited by albumin. When the erythrocyte sediment of the albumin assays was resuspended in VBS-G the cells lysed readily and became large, indicating that complement fixation was not impaired by highly concentrated BSA.

**Swelling of cell ghosts by complement.** Since swelling occurred even in the presence of albumin without apparent osmotic gradient between the intracellular and extracellular compartment, it was suggested that the volume changes of the erythrocytes are caused by a direct effect of complement on the erythrocyte membrane. To test this assumption, we performed the following experiments. In the first set of experiments EA

cells were lysed with distilled water at 4°C and resuspended in VBS-G. Between 30 and 50% of the EA cells could be recovered as ghosts with an electrically measurable mean volume of one-third of the original cell volume. The recovery was not changed by the subsequent incubations. EA cell ghosts in VBS-G were incubated with or without EDTA in whole guinea pig complement. In the absence of EDTA the mean volume of

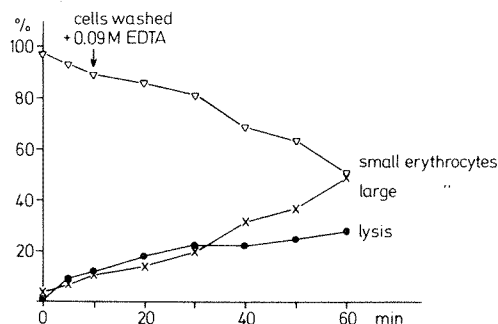


Figure 7. Incubation of EAC1-9hu cells in 0.09 M EDTA buffer at 30°C. EAC1-9hu cells were prepared by incubation of EAC1-8hu cells with C9hu in VBS-G for 10 min at 30°C. The cells were then separated from unbound C9 by centrifugation and resuspended in 0.09 M EDTA buffer at 30°C. Without addition of EDTA the lysis was complete in 30 min. The experiment shows that EDTA prevents lysis but not swelling of the cells.

TABLE I  
Influence of albumin on lysis and volume of EAC1-9 cells

Experiment No.	Incubation of EAC1-8 Cells for 30 min at 37°C	Medium	Lysis	Cell Volume
1		VBS-G	0	% of control 100
2	+ C9	VBS-G	100	140
3		36% BSA in VBS-G	0	100
4	+ C9	36% BSA in VBS-G	10	120
5		Cells of Expt. 4 reincubated in VBS-G	100	140

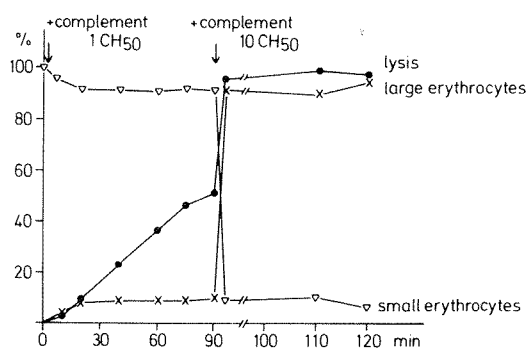


Figure 8. Formation of large erythrocyte ghosts on incubation of complement lysed EA cells with new complement at 37°C. EA cells were first incubated with 1 CH<sub>50</sub> unit of whole guinea pig complement. Approximately 10% large cells were produced under these conditions. After 90 min an additional 10 CH<sub>50</sub> units of complement were added. The remaining erythrocytes lysed within 2 min and approximately 90% of the erythrocyte ghosts became large. Since 50% more lysis leads to 80% more large cells, previously small erythrocyte ghosts swell after the addition of the second complement dose.

the erythrocyte ghosts increased within 60 min and reached the volume of EA cells. In other experiments not shown here even the volume of large cells could be obtained. In the presence of 3 mM EDTA and in the buffer controls (Fig. 9) no significant increase of the mean volume was observed. No hemoglobin was found in the supernatant of the assays and controls, indicating that water lysis had been complete and that the swelling of the erythrocyte ghosts was not due to the colloid osmotic pressure of soluble intracellular protein.

In the second experiment EA cells were lysed by incubating them for 90 min at 37°C with a small amount of guinea pig complement (1 CH50 unit) which generated approximately 10% large cells. When a large dose (10 CH50 units) of guinea pig complement was subsequently added to the same reaction mixture 100% lysis was obtained in approximately 2 min and 90% of the cells became large (Fig. 8), indicating that a substantial number of previously small erythrocyte ghosts had become large by this treatment. Again, protein osmotic forces from the interior of the cell cannot be held responsible for the swelling since the small ghosts were devoid of soluble intracellular protein that was lost during the first incubation period.

#### DISCUSSION

During complement lysis the volume of erythrocytes increases as shown by the volume distribution curves (Fig. 3). For the interpretation of these changes it was important to know if the volume of the erythrocyte ghosts was measured as well as the volume of unlyzed erythrocytes. Since electrical counting showed that the cell concentration in the assay remained unchanged even at 100% lysis (Fig. 2) it is evident that the volume distribution curves represent unlyzed erythrocytes and ghosts. The erythrocyte ghosts retained a high electrical resistance and were impermeable to the electrical current. Since ionic and protein changes occur through the erythrocyte membrane during complement lysis (13, 14), the fact that the electrical current does not permeate the cell may indicate either that the lesions are too small to allow electric permeation or that the cell reseals the lesions immediately after lysis. Resealing would be compatible with electron microscopic and functional observations during the osmotic lysis of erythrocytes (35, 36). A prominent phenomenon in the lytic experiments was the formation of large cells. It occurred with human and guinea pig complement components and with phenantroline as a C9 substitute, likewise. By sequential attachment of the complement components it could be shown that the important step for swelling was the action of C9 or phenantroline on EAC1-8 cells. No changes were observed in

earlier stages of cell intermediates especially not after the attachment of C5 to EAC1-3 cells at which state membrane changes have been observed by others (37). Since a change of erythrocytes to spheroids may falsely be interpreted as a net increase in volume it was necessary to investigate whether a simple change in shape could account for the measured volume increase. A change in shape is reflected by the form factor. The form factor of sheep erythrocytes was determined from photographs (Fig. 1) as 1.27. After complement lysis the erythrocyte ghosts become spheroidal. The form factor for spheroids is 1.50 (31, 32). A spheroid transformation of erythrocytes would account for an increase of 18% in the apparent volume. Since approximately a 40% increase was obtained it can be concluded that the absolute erythrocyte volume increases through the action of complement. In 36% albumin solutions the volume of the erythrocytes increases only by 20% (Table I). This is suggestive of a sphering of the erythrocytes without an increase of the absolute volume.

As shown by a comparison of the mean volume of large and small cells the formation of large cells proceeds as an all or nothing phenomenon. It occurred not only during cell lysis (Figs. 4, 5A, B) but also before actual lysis, namely in the presence of inhibitors (Fig. 7, Table I). Three prelytic stages of EAC1-9 cells could be distinguished. The first stage is fixation of C9 to EAC1-8 cells. This process is temperature independent. It proceeds well at 0°C and does not lead to swelling of the erythrocytes (Fig. 6). The second step consists of swelling of the cells which is temperature dependent. It proceeds in the presence of 0.09 M EDTA (Fig. 7) or 36% albumin (Table I). The third step is the actual lysis of the cells. A temperature-dependent step has been postulated in the terminal stage of lysis. When EAC1-9 cells were preincubated in 0.09 M EDTA at 30°C before lysis they lysed more rapidly than those kept at 0°C (18, 19). Our experiments indicate that this step corresponds to the temperature-dependent swelling of EAC1-9 cells, since it also occurs in 0.09 M EDTA at 30°C (Fig. 7) but not at 0°C. Swelling, therefore, facilitates EAC1-9 cell lysis.

The large cells in lytic experiments are ghosts as can be deduced from the parallelism between increase of hemoglobin in the supernatant and the occurrence of large cells during complement lysis (Fig. 5A). Since it is known that after osmotic lysis the erythrocyte ghosts preferentially reassume their biconcave form (38, 39) the question arose as to why the ghosts would remain large after complement lysis. The maintenance of the biconcave form instead of the energetically more stable spheroid form is ascribed to an as yet unknown structure in the membrane (39). Complement apparently interferes with this structure and thus triggers the all or nothing transition into the spheroid form. In this case swelling would be caused by a process in the membrane rather than by protein osmotic forces from intracellular protein, as is assumed by Green *et al.* (13). This hypothesis is supported by our experimental data. Ghosts of water-lyzed erythrocytes from which the soluble proteins were removed by washing can be specifically swollen by complement (Fig. 9), and erythrocytes lysed with small amounts of complement can be swollen by the addition of new complement (Fig. 8). In both experiments protein osmotic forces do not explain the swelling process. Since the complement action apparently interferes with the maintenance of the biconcave membrane form of the erythrocytes the alteration in the membrane structure seems to be more extensive than the electronmicroscopically visualized limited membrane lesions (2). An indication for structural alterations of the membrane after complement action in addition to the lesions is the

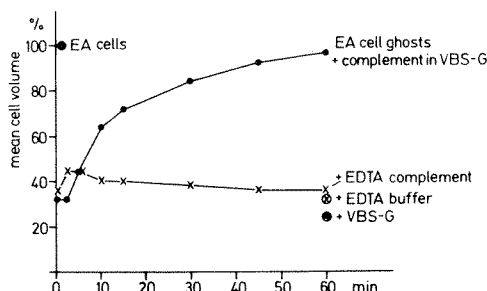


Figure 9. Mean volume of water-lyzed EA cell ghosts incubated with whole guinea pig complement (10 CH<sub>50</sub>) at 30°C in VBS-G buffer with and without 3 mM EDTA. The EA cell ghosts swell specifically by the action of complement.

thickening of the whole membrane in electronmicroscopic sections (2, 40).

With small complement doses only few large cells were formed. The majority of ghosts had the volume of unlyzed cells (Fig. 5B) which is three times higher than that of water-lyzed erythrocytes (Fig. 9). It thus appears that low doses of complement prevent the ghosts from collapsing but are insufficient to trigger the spheroidal transformation. The prevalence of small cells and ghosts suggests that cell swelling is not an absolute prerequisite but facilitates lysis since large cells lyse earlier than small cells (Fig. 5B). In view of the lytic mechanism one explanation of our results would therefore be that lysis is not caused by protein osmotic forces which involve swelling of all cells with final rupture of the cell membrane due to the high intracellular pressure. Recent findings of Lauf (41) suggest that complement lysis can proceed without a classic colloid osmotic swelling phase. An alternative explanation would be the assumption of two swelling mechanisms. The first would be caused by protein osmotic forces. It would be transient and too fast for detection by electrical sizing. After the rupture of the cell, the membrane would reassume its original form. The second mechanism would lead to a permanent swelling of the cell by the direct action of complement on the membrane which is independent of colloid osmotic forces.

*Acknowledgments.* We want to thank Prof. Dr. G. Ruhenstroth-Bauer for his help and interest in this work, Dr. V. Kachel for his advise in photographing particles in the orifice, Dr. V. Bokisch for editorial aid and Ms. G. Franz and S. König for excellent technical assistance.

#### REFERENCES

- Heedman, P. A., *Exp. Cell Res.*, **14**: 9, 1958.
- Humphrey, J. H. and Dourmashkin, R. R., *Adv. Immunol.*, **11**: 75, 1969.
- Humphrey, J. H., *Haematologia*, **6**: 319, 1972.
- Fischer, H., *Ann. N. Y. Acad. Sci.*, **116**: 1063, 1964.
- Smith, J. K. and Becker, E. L., *J. Immunol.*, **100**: 459, 1968.
- Inoue, K. and Kinsky, S. C., *Biochemistry*, **9**: 4767, 1970.
- Lachmann, P. J., Bowyer, D. E., Nicol, P., Dawson, R. M. C., and Munn, E. A., *Immunology*, **24**: 135, 1973.
- Kinsky, S. C., Luse, S. A., and van Deenen, L. L. M., *Fed. Proc.*, **25**: 1503, 1966.
- Seeman, Ph., *Fed. Proc.*, **33**: 2116, 1974.
- Mayer, M. M., *Proc. Natl. Acad. Sci.*, **69**: 2954, 1972.
- Kolb, W. P., Haxby, J. A., Arroyave, C. M., and Müller-Eberhard, H. J., *J. Exp. Med.*, **135**: 549, 1972.
- Kolb, W. P. and Müller-Eberhard, H. J., *Fed. Proc.*, **33**: 796, 1974.
- Green, H., Barrow, P., and Goldberg, B., *J. Exp. Med.*, **110**: 699, 1959.
- Hingson, D. J., Massengill, R. K., and Mayer, M. M., *Immunochimistry*, **6**: 295, 1969.
- Lauf, P. K. and Dessent, M. P., *Immunol. Commun.*, **2**: 193, 1973.
- Lauf, P. K., *BBA, Biochem. Biophys. Acta*, **415**: 173, 1975.
- Leddy, J. P., Thiem, P. A., Leblond, P. F., Weed, R. I., and Lauf, P. K., *J. Immunol.*, **108**: 475, 1972.
- Frank, M. M., Rapp, H. J., and Borsos, T., *J. Immunol.*, **93**: 409, 1964.
- Frank, M. M., Rapp, H. J., and Borsos, T., *J. Immunol.*, **94**: 295, 1965.
- Kaliner, M. and Austen, K. F., *Science*, **183**: 659, 1974.
- Cooper, N. R., Polley, M. J., and Oldstone, M. B. A., *J. Immunol.*, **112**: 866, 1974.
- Rapp, H. J. and Borsos, T., *Molecular Basis of Complement Action*, Appleton Century Crofts, New York, 1970.
- Shin, H. S. and Mayer, M. M., *Biochemistry*, **7**: 2997, 1968.
- Colten, H. R., Bond, H. E., Borsos, T., and Rapp, H. J., *J. Immunol.*, **103**: 862, 1969.
- Polley, M. J. and Müller-Eberhard, H. J., *J. Exp. Med.*, **126**: 1013, 1967.
- Kachel, V., Metzger, H., and Ruhenstroth-Bauer, G., *Z. Gesamte. Exp. Med.*, **153**: 331, 1970.
- Thom, R., in *Modern Concepts in Hematology*, Edited by G. Izak and S. M. Lewis, p. 191, Academic Press, New York, 1972.
- Hofmann, H., Program Library, Max-Planck-Institut für Biochemie, D-0833 Martinsried, Germany.
- Metzger, H., Valet, G., Kachel, V., and Ruhenstroth-Bauer, G., *Blut*, **25**: 279, 1972.
- Kachel, V., *Microsc. Acta*, **75**: 419, 1974.
- Hurley, J., *Biophys. J.*, **10**: 74, 1970.
- Grover, N. B., Naaman, J., Ben-Sasson, S., and Doljanski, F., *Biophys. J.*, **9**: 1398, 1969.
- Hadding, U. and Müller-Eberhard, H. J., *Science*, **157**: 442, 1967.
- Ruhenstroth-Bauer, G., Valet, G., Kachel, V., and Boss, N., *Naturwissenschaften*, **61**: 260, 1970.
- Seeman, Ph., *J. Cell Biol.*, **32**: 55, 1967.
- Bodemann, H. and Passow, H., *J. Membr. Biol.*, **8**: 1, 1972.
- Polley, M. J., Müller-Eberhard, H. J., and Feldman, J. D., *J. Exp. Med.*, **133**: 53, 1971.
- Passow, H., in: *Hämolyse-hämolysische Erkrankungen*, Edited by L. Nowicki, H. Martin, and J. C. F. Schubert, p. 37, Lehmanns Verlag, München, 1973.
- Weinstein, R. S., in *The Red Blood Cell*, Edited by D. M. N. Surgenor, Vol. 1, p. 213, Academic Press, New York, 1974.
- Polley, M. J., *Prog. Immunol.*, **1**: 597, 1971.
- Lauf, P. K., *Z. Immunitätsforsch.*, **147**: 314, 1974.