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HEMOLYSIS OF RABBIT ERYTHROCYTES BY PURIFIED STAPHYLOCOCCAL ALPHA-TOXIN

III. POTASSIUM RELEASE

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ABSTRACT

MADOFF, MORTON A. (New England Center Hospital, Boston, Mass.), LOUIS Z. COOPER, AND LOUIS WEINSTEIN. Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. III. Potassium release. J. Bacteriol. 87:145-149. 1964.—The reaction between staphylococcal alpha-toxin and erythrocytes was characterized by rapid release of K^+ from the cells, early in the prelytic period; 50 to 75% of this loss occurred before leakage of hemoglobin was detectable. The addition of specific antitoxin early enough in the reaction to inhibit gross hemolysis also inhibited cation release. The presence of sucrose or polyethylene glycol prevented hemoglobin release, but was without effect on K^+ leak. These observations suggest that K^+ loss is a more specific indication of the progress of the reaction between alpha-toxin and erythrocytes than is the release of hemoglobin.

Prelytic release of potassium ion was documented in a wide variety of hemolytic systems which share the characteristics of "colloid osmotic swelling" (Davson and Danielli, 1938; Ting and Zirkle, 1943; Green, Barrow, and Goldberg, 1959; Jacob and Jandl, 1962; Wilbrandt, 1941). Previous studies (Cooper, Madoff, and Weinstein, 1964*a*, *b*) indicated that the hemolysis of rabbit erythrocytes induced by highly purified staphylococcal alpha-toxin also fits this common pattern. The course of alpha-lysin hemolysis was demonstrated to consist of a prelytic lag followed by a period of rapid hemolysis; addition of sucrose or polyethylene glycol (PEG) prevented release of hemoglobin, even when added during the period of rapid lysis; specific antiserum, on the other hand, had to be added early in the prelytic phase to prevent laking of the red cells. When toxin-treated erythrocytes were removed from a sucrose-containing suspension and resuspended in phosphate-buffered saline (PBS), they lysed as if they had not been exposed to sucrose. Conversely, the inhibition of hemolysis by antitoxin could not be reversed when the cells were placed in PBS. These studies suggested that the lysin-erythrocyte reaction was essentially completed in the prelytic period, that this reaction could be prevented by antiserum but not by an osmotic stabilizer, and that the release of hemoglobin which followed could be prevented or delayed when cells were suspended in sucrose or PEG.

The purpose of the present paper is to report the results of studies of release of K^+ from lysin-treated erythrocytes. This was found to occur in the prelytic phase of the reaction, and to be inhibited only by specific antitoxin but not by osmotic stabilizers.

MATERIALS AND METHODS

The methods of the production and purification of staphylococcal alpha-lysin and of the study of the time-course of hemolysis were described previously (Cooper et al., 1964*a*, *b*; Madoff and Weinstein, 1962). Rabbit erythrocytes, obtained fresh daily in Alsever's solution, from the central artery of the ear were washed twice in 0.155 M sodium chloride (normal saline) and suspended in PBS which contained 0.145 M sodium chloride and 0.01 M phosphate, buffered at pH 6.9. A 1.5 M sucrose solution in PBS was used. PEG was prepared by adding one part solid Carbowax 4000 (Union Carbide Chemical Co., New York, N.Y.) to seven parts liquid PEG (molecular weight, 300). This was solid at room temperature, but liquid at 37 C after being melted in a boiling-water bath. Alpha-lysin antiserum (horse), obtained from Wellcome Research Laboratories, Beckenham, England, and containing 900 units of antihemolysin per ml, was heated at 56 C for 30 min prior to use.

Hemoglobin release was measured by deter-

mining the optical density of 1 ml of supernatant fluid, appropriately diluted in a 1% sodium carbonate solution, at 541 mμ in a Coleman Junior spectrophotometer. A sample of the solution, diluted in lithium hydroxide, was used for K^+ determination in a Baird-Atomic DB flame photometer.

RESULTS

Effect of antitoxin on prelytic release of K^+. Portions (1 ml) of purified alpha-lysin (3,000 hemolytic units per ml) diluted 1:750 in PBS were added to each of four rows of test tubes and prewarmed to 37 C in a shaker bath; next, 0.75-ml portions of a preheated 8% suspension of rabbit erythrocytes were added to each tube at time 0. A tube was removed from each row at selected intervals, and immediately centrifuged at 1,000 × *g* for 2 min at 4 C. Per cent hemolysis and K^+ concentration were measured with 1.0 ml of the supernatant fluid. A few seconds after time 30 sec, 0.25 ml of antitoxin was added to each of the remaining tubes in the first row, and after 1.5 and 3 min to those remaining in the second and third rows, respectively. Antitoxin was not added to the fourth row. Hemoglobin and K^+ concentrations were also measured in controls incubated under the same conditions. K^+ release from ery-

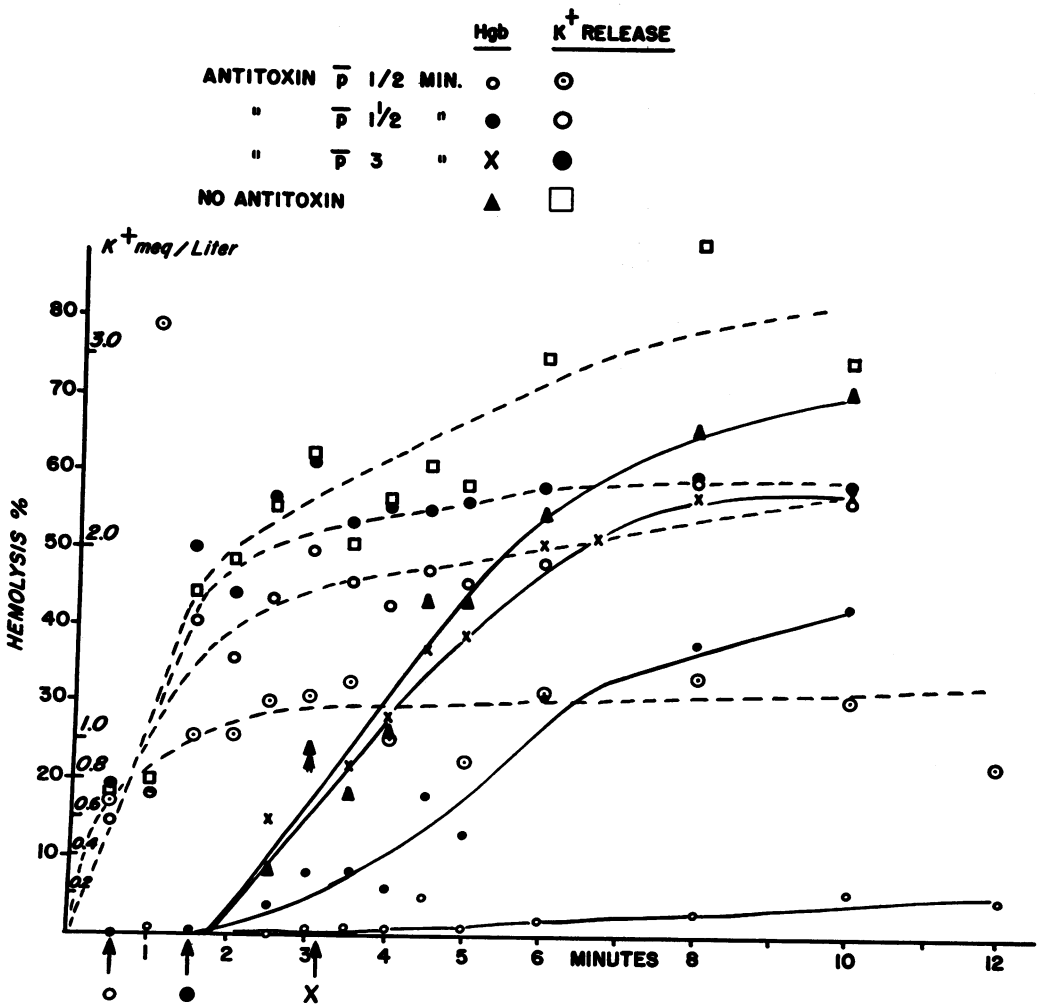

FIG. 1. *Effect on hemolysis and K^+ release of alpha-lysin antiserum added during different phases of the hemolytic reaction.*

throcytes suspended in PBS was minimal during the brief incubation period; the addition of the antitoxin was without effect on K^+ release.

The hemolytic curve of the controls was of the expected shape, with a clearly defined preltyic phase which lasted for 2 to 2.5 min, followed by a period of rapid lysis which tailed off asymptotically (Fig. 1). The addition of antitoxin after 30 sec suppressed hemolysis almost completely; addition of antiserum after longer intervals was less effective, so that, after 3 min, hemoglobin release was almost unaffected.

Potassium ion release was noted in all samples taken after 30 sec; the addition of antitoxin after 30 sec and 1.5 and 3 min produced marked inhibition of K^+ release at successively higher plateaus, directly related to the duration of the delay before exposure of the system to antibody. The results of this study delineated several features of the alpha-lysin–erythrocyte reaction. K^+ release began in the earliest portion of the "prelytic period" and proceeded at such a rapid rate that over half of the total loss of this ion from the cells occurred before hemoglobin was detectable in the supernatant fluid. The release of K^+ appeared to be an intrinsic part of alpha-lysin hemolysis, since suppression of hemoglobin release by antitoxin also prevented leakage of the cation.

Effect of sucrose and PEG on prelytic release of K^+. With the procedure described above, 0.25 ml of 1.5 M sucrose was substituted for the antitoxin. The time intervals at which sucrose was added were 1, 3, and 7 min in the first, second, and third rows, respectively. The carbohydrate produced marked inhibition of hemoglobin release (Fig. 2). The pattern of the reaction was very different from that observed with antitoxin. Addition of sucrose, even during rapid lysis, stopped hemoglobin release abruptly. The earlier the sugar was added, the more prolonged was the inhibitory effect.

Sucrose produced no essential alteration in re-

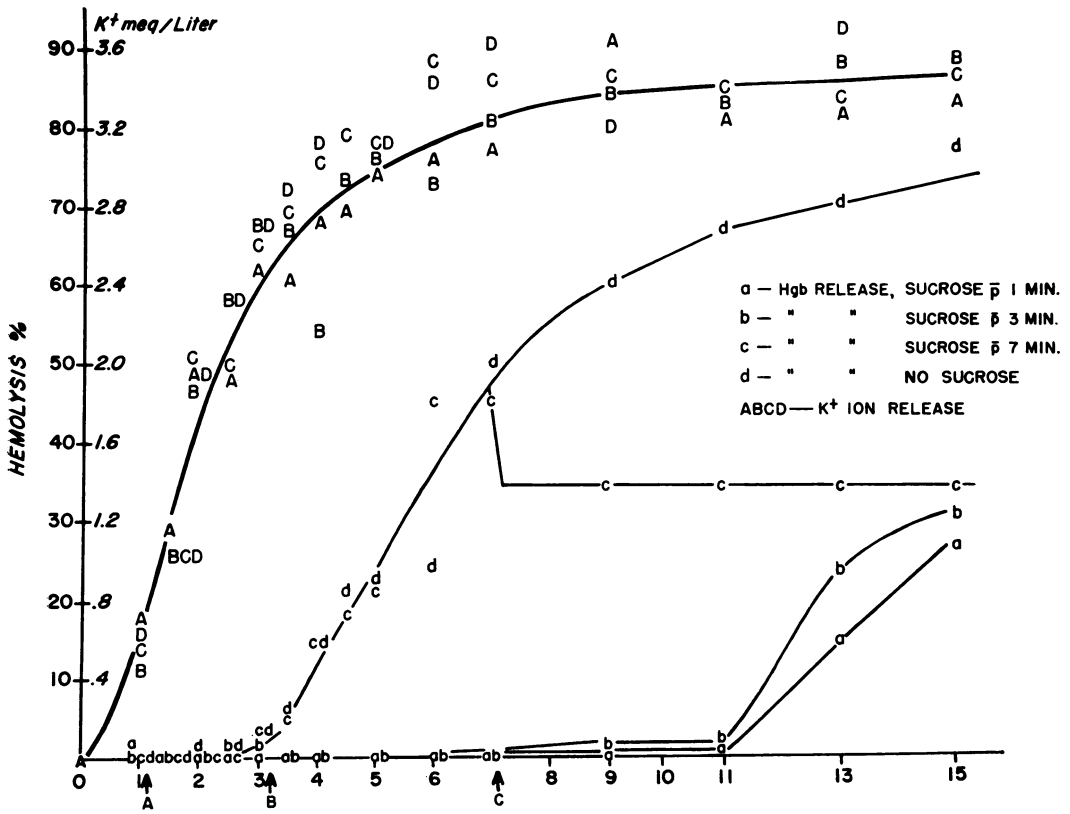

FIG. 2. *Effect on hemolysis and K^+ release of sucrose added during various phases of the hemolytic reaction.*

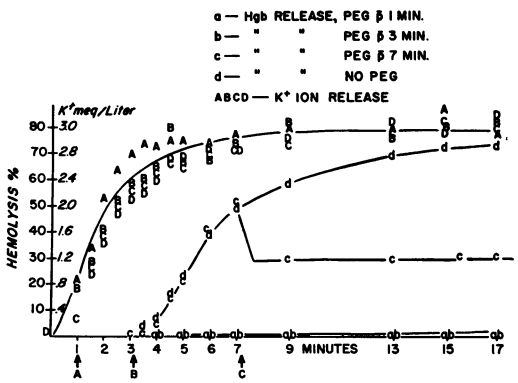

FIG. 3. *Effect on hemolysis and K^+ release of polyethylene glycol added during various phases of the hemolytic reaction.*

lease of K^+, despite its effect on gross hemolysis. Correction of the values for cation loss in the controls, to account for dilution due to addition of the sugar to the experimental tubes, would show the K^+ release curves to be identical, within the limits of experimental error.

This experiment was repeated with 0.25 ml of PEG instead of sucrose. The results (Fig. 3) demonstrated the effectiveness of PEG in preventing further release of hemoglobin even when it was added during the phase of rapid lysis. As with sucrose, however, K^+ release was unimpaired.

DISCUSSION

The results of this study support the previously proposed concept (Cooper et al., 1964*b*) that the action of staphylococcal alpha-lysin on the erythrocyte is limited to the "prelytic period." Potassium ion leaks rapidly from the cells after they are exposed to toxin, so that 50 to 75% of the total loss occurs before any hemoglobin release is detectable. The premise that this leakage is a direct result of the activity of the lysin on the red-cell membrane is supported by the observation that the presence of specific antiserum early in the prelytic phase inhibits K^+ release. The effects noted with sucrose and PEG are consistent with the commonly proposed theory that lysin activity opens "pores" in the erythrocyte membrane. This is thought to permit leakage of ions of low molecular weight across the membrane in both directions, leading to cell swelling as water follows the inflow of salt. This results in "stretching" of the "opened pores" to a size sufficient to allow loss of large molecules, specifically hemoglobin. Sucrose and PEG, which are unable to penetrate the cell membrane, prevent swelling by virtue of their colligative properties. This theory of "colloid osmotic swelling" (Wilbrandt, 1941) was found to be applicable to a large variety of hemolytic phenomena (Davson and Danielli, 1938; Ting and Zirkle, 1943; Green et al., 1959; Jacob and Jandl, 1962; Wilbrandt, 1941; Bernheimer, 1947). Since sucrose is only temporarily effective in preventing hemoglobin release, it may be postulated that the alpha-lysin–damaged membrane gradually becomes permeable to the sugar and that, as it does, the effect of high external osmotic pressure is lost.

The possibility that alpha-lysin interferes with the K^+ pumping mechanism (Maizels, 1951; Glynn, 1956; Hoffman, 1962) merits mention. Jacob and Jandl (1962), in their discussion of hemolysis by sulfhydryl inhibitors, discarded this theory because the rate of K^+ loss was four to five times greater than that of K^+ exchange in the normal cell (Solomon, 1952). The loss of K^+ resulting from exposure of erythrocytes to staphylococcal alpha-lysin is even more rapid than that produced by sulfhydryl inhibitors.

The similarity between the results of the studies described in this paper and those reported by Bernheimer (1947) in an investigation of *Clostridium septicum* toxin and by Green et al. (1959) in studies of antibody-complement hemolysis is striking.

Although the data presented in this and previous reports (Cooper et al., 1964*a*, *b*) indicated certain of the steps involved in the lysis of erythrocytes by staphylococcal alpha-lysin, the exact mechanisms responsible for these phenomena are still unknown.

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