Rickettsial Hemolysis: Adsorption of Rickettsiae to Erythrocytes

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The hemolysis of sheep red blood cells by Rickettsia prowazekii has been characterized. A requirement for the adsorption of the rickettsia to the erythrocyte was indicated. No indication of the production of a soluble hemolysin was observed. Neither adsorption nor hemolysis can proceed at 0 C. The hemolytic process is rapid with a half-time of less than 30 min, and both the rate and extent are proportional to the number of rickettsiae present.

The typhus rickettsiae, Rickettsia prowazekii and R. mooseri, are able to lyse the erythrocytes of several species. This ability has been directly correlated by Bovarnick and others (1, 2, 4) with both the infectivity of the rickettsiae as measured by the lethal doses for eggs and mice and their metabolic activity as measured by oxidative phosphorylation. The rapidity and reliability of the hemolytic assay makes it a very valuable tool for the quantitation of the biological activity of an intracellular parasite whose sluggish metabolism and difficult cultivation makes other assays laborious and time consuming.

Furthermore, an understanding of the mechanism of hemolysis will offer valuable insights concerning the interaction of the rickettsiae with its host cells. However, with the exception of the work of Clarke and Fox (6) in 1948, nothing of the mechanism of hemolysis is known. Clarke and Fox had postulated that the rickettsiae released a soluble toxin which brought about the lysis of the erythrocyte.

In this investigation, portion of a thesis submitted to the University of Virginia in partial fulfillment for a M.S. degree, we have characterized the interaction of the rickettsiae and erythrocytes and shown that, contrary to the postulate of Clarke and Fox, an adsorption of the rickettsiae to the red blood cell is necessary for hemolysis.

MATERIALS AND METHODS

Rickettsial preparation and growth. R. prowazekii, Madrid E strain, was propagated in 6-day embryonated antibiotic-free hen’s eggs by inoculation of 0.2 ml of a 10^-4 dilution of a seed pool. The seed pool was prepared from lyophilized material (yolk sac passage 270). Infected yolk sacs were harvested 8 to 9 days postinoculation and stained for rickettsiae according to the method of Gimenez (7). Rickettsial suspensions were prepared from heavily infected yolksacs.

The purification procedure, carried out at 4 C, was a modification of the methods of Bovarnick (5) and Wisseman (11). Twenty percent (w/v) suspensions of infected yolksacs in a sucrose-phosphate-glutamate (SPG) buffer were homogenized for 1 min in a Waring blender. The suspension was centrifuged at 600 x g for 10 min. The top fatty layer and pellet were discarded, and the supernatant fluid (middle layer) was centrifuged at 12,000 x g for 10 min. The pellet from this centrifugation was homogenized and resuspended to a volume equivalent to the original 20% suspension. This suspension was further purified by the addition of 1 g of acid-washed Celite per 6 g of original yolksac (Hyflo super Cel, Fisher) and bovine serum albumin (Fraction V, Sigma or Schwartz-Mann) to obtain a final concentration of 0.6%. This mixture was centrifuged at 121 x g for 10 min to sediment the Celite and any precipitated material. The resulting supernatant fluid was centrifuged at 12,000 x g for 10 min. The pellet from this centrifugation was resuspended to a volume equivalent to the original 20% suspension and centrifuged at 121 x g for 10 min, and the resulting supernatant fluid was centrifuged at 12,000 x g for 10 min. The pellet from this centrifugation was resuspended, usually to an 80% suspension, that is, a volume equivalent to one-fourth of the original volume. This suspension was centrifuged for 5 min at 200 x g to sediment any aggregated material or remaining Celite. This final “80% suspension” was the purified material used in this investigation. Only fresh rickettsial material was used since other investigators had shown that a freeze-thaw procedure had adverse effects on rickettsial hemolytic activity (10).

Materials. The diluent for the rickettsial inoculum and rickettsial suspension in the purification procedure was an SPG solution originally devised by Bovarnick (3), consisting of 0.218 M sucrose, 0.00376 M KH2PO4, 0.0071 M KH2PO4, and 0.0049 M glutamic acid, pH 7. The diluent for the sheep red blood cells was SPG-Mg, containing 0.01 M MgCl2 in the SPG. During the purification procedure, 6% bovine serum
albumin in equal portions of 0.122 M KCl and 0.023 M NaCl, pH 7, (3) was added to the homogenate. Washed sheep red blood cells for hemolytic assays were prepared in SPG-Mg according to Snyder and Bovarnick (10).

Hemolytic assay. The hemolysis tests used are modifications of the method of Snyder and Bovarnick (10). In the routine assay, the rickettsial suspension to be assayed was diluted in twofold serial dilutions in SPG. A portion of each dilution (0.2 ml) was incubated with 0.4 ml of 25% sheep red blood cells at 34 C for 150 min. The reaction was terminated by the addition of 0.85% NaCl containing Formalin (0.2 ml per 100 ml of NaCl), and the mixtures were centrifuged at 733 x g for 10 min. The amount of released hemoglobin (in optical density [OD] units) was measured spectrophotometrically at 545 nm. Controls for spontaneous lysis and rickettsial turbidity were included, and the values obtained were usually subtracted from the 545-nm readings.

Protein assays. Protein was determined by the method of Lowry et al. (8).

Electron microscopy. Samples for electron microscopy were fixed in glutaraldehyde and OsO4 and stained with uranyl acetate and lead citrate according to the method of Schnaitman and Greenawalt (9).

Separation of adsorbed and unadsorbed rickettsiae in the hemolytic system. In many experiments, the rickettsiae-red blood cell system was separated by centrifugation to assay the fractions for hemolytic activity. The rickettsiae and red blood cells were mixed in the usual 1:2 ratio, and, prior to incubation, a 0.6-ml sample was removed which yielded a measure of the total hemolytic capacity of the system. After the desired incubation period, the mixture was centrifuged at 450 x g for 7 min to sediment the red blood cells and adsorbed rickettsiae but not rickettsiae free in suspension. The resulting supernatant fraction was assayed to determine the extent of hemolysis during the incubation period by adding 0.6-ml samples to 2 ml of 0.85% NaCl with Formalin and reading at 545 nm. The supernatant fraction was also assayed for remaining hemolytic activity due to unadsorbed rickettsiae by adding to 0.4 ml of red blood cells to 0.2 ml of supernatant suspension. The red blood cell-adsorbed rickettsiae pellet was resuspended to the original volume with SPG-Mg, incubated, and assayed for hemolytic activity. All of the samples were incubated at 34 C, usually for 150 min. At the end of incubation, 2 ml of 0.85% NaCl with Formalin was added to stop the reaction, and the samples were mixed and centrifuged at 733 x g for 10 min. The released hemoglobin was read at 545 nm.

RESULTS

Time course for hemolysis. The hemolysis of sheep red blood cells as a function of time was examined over an 18-hr period. As shown in Fig. 1 and 2 the reaction presented a hyperbolic curve in which more than 50% of the hemolysis occurred during the first 30 min at 34 C. At 150 min, the time chosen by Snyder and Bovarnick (10) for their short hemolysis test, the reaction had reached a plateau with less than a doubling occurring if the incubation period was extended for 18 hr. (Fig. 2). The dashed line in Fig. 2 shows that there was little spontaneous hemolysis over the 18 hr.

Effect of rickettsial and erythrocyte concentration. In the presence of an excess of red blood cells the amount of hemolysis was a function of the concentration of biologically active rickettsiae. Figures 1 and 3 demonstrate that there was a linear relationship between hemolysis and rickettsial concentration over an eightfold range. A change in rickettsial concentration produced no change in the time course so that linearity was obtained whether the hemolysis was measured after 30, 60, or 150 min. The amount of hemoglobin release was the same using 12.5 or 25% red cells. In both cases the red cells were in excess since complete hemolysis of 12.5% red blood cells would have resulted in an absorbance at 545 nm of 3.49, greater than that observed with the highest rickettsial concentration employed. The hemolytic system was independent of the total volume of the assay. Without changing the 1:2 ratio of rickettsiae-to-red blood cells, the volume was increased 50-fold without change in the hemolytic activity (data not shown).

Effect of temperature. Previous investigations (6) have shown that hemolysis will not occur at 0 C. We have confirmed this observation (Table 1 and Fig. 4). The small absorbance value observed can be accounted for by spon-
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Table 1. Hemolytic activity at various temperatures

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Hemolytic activity* (absorbance 545 nm)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.00, 0.00</td>
</tr>
<tr>
<td>15</td>
<td>0.10, 0.26</td>
</tr>
<tr>
<td>25</td>
<td>0.48, 0.64</td>
</tr>
<tr>
<td>34</td>
<td>0.83, 0.88</td>
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*Rickettsial suspensions were incubated with 25% red blood cells for 150 min. Results of two experiments are shown.

![Graph](image1.png)

Fig. 2. Eighteen-hour time course of hemolysis at 34°C. Rickettsial suspensions were incubated at 34°C with 25% red blood cells. Three curves, dilutions of one rickettsial suspension; dashed line, the red blood cell control without rickettsiae.

![Graph](image2.png)

Fig. 3. Effect of rickettsial concentrations on hemolytic activity. Various concentrations of a rickettsial suspension are plotted against the amount of hemolytic activity (absorbance at 545 nm) obtained after incubations at 34°C for 30 min, 60 min, and 150 min. Data are from Fig. 1.

![Graph](image3.png)

Fig. 4. Effect of preincubation at 0°C on hemolysis. Twenty percent rickettsial suspensions were mixed with 12.5% red blood cells at 0°C, and 0.6-ml samples were dispensed into test tubes at 0°C for 0 min (■), 15 min (▲) or 30 min (▼) and then switched to 34°C as indicated. Dashed line, sample maintained at 0°C throughout.

taneous lysis and the turbidity of the rickettsiae. Furthermore, as shown in Fig. 4, there was no “early” temperature independent process that could be detected. Preincubation of the suspension of 0°C for 30 min neither altered the time course nor the amount of hemoglobin released in a subsequent incubation at 34°C. Likewise, as shown in Fig. 5, there was no “late” step in hemolysis that could proceed at 0°C. The hemolytic reaction at 34°C could be stopped at all times indicated without subsequent hemolysis occurring by lowering the temperature to 0°C. As shown in Table 1 intermediate temperatures allowed hemolysis to proceed at a reduced rate. After 150 min, the hemolysis which occurred at 15 or 25°C had not reached the plateau value found for hemolysis at 34°C.

Adsorption of rickettsiae to red blood cells. To determine whether the rickettsiae adsorbed to the sheep red blood cells to initiate hemolysis or whether a soluble hemolysin (such as the toxin proposed by Clarke and Fox [6]) was released, the rickettsiae and red blood cells were separated after the initiation of hemolysis. Centrifugation of a rickettsial suspension at 455 × g for 7 min did not sediment the rickettsiae; however, under these conditions...
the red blood cells were sedimented (Table 2). Thus, if the hemolytic mixture of rickettsiae and red blood cells was centrifuged under these conditions, free, unadsorbed rickettsiae would be in the supernatant fraction, whereas adsorbed rickettsiae would be sedimented with the red blood cells. Table 3 shows several experiments in which the adsorbed and free rickettsiae were separated by this centrifugation procedure after the hemolytic reaction had proceeded for 10 or 30 min. Recoveries of the initial (before centrifugation) hemolytic activity ranged from 50 to 100% with a mean of 86%. Recovery was calculated by recombining the supernatant and sedimented fractions and incubating the recombined parts for 150 min at 34 C. Recovery, as measured by the sum of the hemolytic activities of the independently assayed fractions, was in excellent agreement and had a range of 58 to 144% of the initial activity with a mean of 92%. When the hemolytic suspension had been incubated at 0 C, a condition during which no hemolysis could occur, essentially all (greater than 88%) of the hemolytic activity recovered was in the supernatant, unadsorbed rickettsiae, fraction. However, at 34 C the hemolytic activity was not found in the supernatant fraction. At 34 C, 71 to 100% of the hemolytic activity was sedimentable with red blood cells, indicating that the rickettsiae had adsorbed under these conditions. The absence of hemolytic activity in the supernatant fluid at 34 C was not due to preferential inactivation of the rickettsiae at 34 C relative to 0 C. Less than 10% of the hemolytic activity of the rickettsia was lost by a 10-min incubation at 34 C in the absence of red blood cells.

Observations of the sedimentable fraction by electron microscopy confirmed that the rickettsiae were adsorbed to the erythrocytes at 34 C, when hemolysis occurred, but were unadsorbed at 0 C, when there was no hemolytic reaction. Rickettsiae and red blood cells were incubated and centrifuged as in Table 3. Fixed and stained thin sections of the sedimented material were examined. As shown in Table 4 and Fig. 6, rickettsiae were present in the sections in which the hemolytic mixture had been incubated at 34 C, but no rickettsiae were seen when the incubation temperature had been 0 C. The sections derived from the sample incubated at 34 C had 10 rickettsiae per 100 red blood cells. Sections prepared from the sedimented material which had been extensively washed in SPG three times had 5 rickettsiae per 100 red blood cells. This indicated that the rickettsiae were not simply trapped with the red blood cells at 34 C and that a temperature-sensitive adsorption of the rickettsia to the erythrocyte was an early and necessary part of the hemolytic reaction. Because of the difficulties in quantitation of particles in thin section, the numbers are given no significance other than that rickettsiae were present when hemolysis occurred and absent when there was no hemolysis.

In Table 2, rickettsiae were not sedimented by low-speed centrifugation.

<table>
<thead>
<tr>
<th>Centrifugal procedure</th>
<th>Hemolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>None</td>
<td>0.48</td>
</tr>
<tr>
<td>455 × g, 7 min</td>
<td>0.50</td>
</tr>
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After the indicated centrifugation of a rickettsial suspension, the supernatant suspension was added to 25% red blood cells and incubated for 150 min at 34 C.

DISCUSSION

At the onset of this investigation, three models for the mode of hemolysis of sheep red blood cells by *R. prowazekii* seemed apparent: (i) the excretion of a soluble hemolytic exotoxin, (ii) a hemolytic factor bound to the surface of the rickettsia, or (iii) an abortive attempt by the rickettsia to parasitize the erythrocyte.

Clarke and Fox (6) had investigated the hemolysis of rabbit erythrocytes by murine typhus rickettsiae. They concluded that a toxin was released by the rickettsiae that was both soluble and labile. The basis for these conclusions was the failure to demonstrate adsorption of rickettsiae to the red blood cells and the fail-
To sustain the soluble hemolysin argument would require that the hemolysin be produced or released in a temperature-sensitive fashion only when red cells are present. Since no hemolytic activity, either particulate or soluble, can be found in this supernatant fraction of the hemolytic system, one also would have to argue that the soluble hemolysin was a one-shot system. Once the hemolysin was released from the rickettsiae, it would have to adsorb to the red cell, and no further hemolysin could be released from the rickettsiae in the presence of new red blood cells. Furthermore, the observation that both the rate and extent of hemolysis are proportional to the number of rickettsiae added to an excess of red blood cells suggests that the hemolytic agent acts in a stoichiometric fashion. If a soluble hemolysin were released, one would expect that, as the period of incubation was increased, hemolysis would continue until all erythrocytes were lysed. Similarly, if a rickettsia could lyse one erythrocyte after another, complete hemolysis should be observed, as well as a striking dependence on red blood cell concentration. The data are to the contrary: neither complete hemolysis nor a marked dependence on erythrocyte concentration (as long as they were in excess) was observed. And lastly, the electron micrographs show that rickettsiae are associated with the red cells if, and only if, conditions are suitable for hemolysis to ensue.

Rickettsial hemolysis differs greatly from another hemolytic system that requires adsorption, viral hemolysis. The hemolysis caused by rickettsiae is very temperature dependent, and neither hemolysis nor adsorption will occur at 0°C. Thus, adsorption of rickettsiae is not a passive event but presumably requires metabolic activity on the part of the rickettsiae or

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Initial hemolytic activity (absorbance)</th>
<th>Hemolytic activity (% of initial)</th>
<th>Ratio of hemolytic activity in the sedimented fraction to sum of sedimented plus supernatant fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Recovery</td>
<td>Supernatant fraction (free rickettsiae)</td>
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<tr>
<td>Temp (C)</td>
<td>Time (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
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<td>1.80</td>
<td>86</td>
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<td>34</td>
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</tr>
<tr>
<td>34</td>
<td>30</td>
<td>1.39</td>
<td>106</td>
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* Rickettsiae and red blood cells were incubated and centrifuged at 455 × g, 7 min, and the pellet was washed by resuspending and recentrifuging where indicated. Thin sections were examined for the presence of red blood cells and rickettsiae.

To recapitate our evidence against a soluble hemolysin and supporting an adsorption step, no soluble hemolysin can be found in the medium after incubation of the rickettsiae at 34°C, and little loss of hemolytic activity associated with the rickettsiae was observed during this incubation. Hemolytic activity is quickly removed from the supernatant fraction after mixing and centrifuging the hemolytic system of erythrocytes and rickettsiae at 34°C; however, at 0 or 34°C without red blood cells all hemolytic activity is retained in the supernatant.
FIG. 6. Electron micrograph of sheep red blood cells with adsorbed typhus rickettsiae. Thin sections of the sedimented fraction were obtained after preincubation of rickettsiae and red blood cells for 10 min at 34°C and centrifugation at 455 × g for 7 min. Bar markers: top, 1 μm; bottom, 0.1 μm.
erythrocyte. Similarly, hemolysis, once established by the adsorption of the rickettsia, requires continued metabolism and will not proceed at 0 C.

Therefore, although a choice between a membrane-bound hemolysin and abortive parasitism (models 2 and 3) cannot be definitely made on the basis of available data, the hemolytic process is certainly very complex and requires the continuance of reactions which have a high activation energy. This could equally well be an abortive attempt to parasitize the erythrocyte with a failure to reseal the atypical erythrocyte membrane or an enzymatic lysis of this membrane by enzymes on the surface of the rickettsia. We are attempting to further investigate the nature of these metabolic requirements.

ACKNOWLEDGMENTS

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LITERATURE CITED


