Rickettsial Hemolysis: Effect of Metabolic Inhibitors upon Hemolysis and Adsorption

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The hemolysis of sheep red blood cells by typhus rickettsiae is initiated by a temperature-sensitive adsorption process that is dependent on the energy-generating metabolism of the rickettsiae but not that of the erythrocyte. Adsorption is followed by events dependent on the metabolism of both the rickettsiae and red blood cells. At 34 C the adsorption step is complete after about 4 min, at lower temperatures the time required for adsorption is increased. At 0 C no adsorption occurred. The addition of cyanide inhibited the catabolism of glutamate by the rickettsiae and, consequently, both adsorption and the subsequent steps in hemolysis. The starvation of the rickettsiae for glutamate also prevented adsorption. Fluoride had no demonstrable effect on rickettsial metabolism nor the adsorption step but inhibited glycolysis in the erythrocytes and the hemolysis of the erythrocytes by rickettsiae.

It has been known for several years that the ability of *Rickettsia prowazekii* to lyse sheep red blood cells exhibits, with some notable exceptions, the same activation and inactivation profiles as rickettsial respiration, phosphorylation, and infectivity (1, 2, 11). For example, Snyder and Bovarnick (11) showed that cyanide would inhibit both respiration and hemolysis. Fluoride, however, had no effect on rickettsial respiration but would inhibit hemolysis. This suggested that the erythrocyte's metabolism was also important for hemolysis. We have recently presented evidence that the first step in the hemolytic scheme is a temperature-sensitive adsorption of the rickettsia to the erythrocyte (9). No soluble hemolysin was released, and the adsorption step was not a passive one such as observed with viral hemolysis. A complex process dependent on the metabolism of the cells involved (for example, either enzymatic lysis of the erythrocyte membrane or an abortive attempt by the rickettsia to parasitize the erythrocyte) was suggested by these data. In the present investigation, we describe the effects of inhibiting the energy-generating metabolism of the rickettsia and erythrocyte on both the overall process of hemolysis and on the adsorption step. An understanding of the metabolic requirements of the hemolytic reaction may be useful in deciphering the mechanism by which rickettsia enters its host cell and also provides a tool for the investigation of the biological activities of this organism. (This work represents a portion of a thesis submitted by L.E.R. to the University of Virginia in partial fulfillment of the requirements for the degree of Master of Science.)

MATERIALS AND METHODS

Rickettsial preparation and growth. *R. prowazekii*, Madrid E strain, was propagated in 6-day embryonated, antibiotic-free hen eggs by inoculation with 0.2 ml of a 10^{-5} dilution of a seed pool. The seed pool was prepared from lyophilized material (yolk sac passage no. 270). Infected yolk sacs were harvested 8 to 9 days postinoculation and stained for rickettsiae by the method of Gimenez (7). Rickettsial suspensions were prepared from heavily infected yolk sacs. The purification procedure was carried out at 4 C by a modification (9) of the methods of Bovarnick (4) and Wisseman (12). The final "80% suspension" obtained by this procedure 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 (11).

Materials. The diluent for the rickettsial inoculum and rickettsial suspension in the purification procedure was a sucrose-phosphate-glutamate (SPG) solution originally devised by Bovarnick (3). The diluent for the sheep red blood cells was SPG-Mg, containing 0.01 M $MgCl_2$ in the SPG. Washed sheep red blood cells for hemolytic assays were prepared in SPG-Mg by the method of Snyder and Bovarnick (11). Glutamate- $U-^{14}C$ was obtained from New England Nuclear Corp.

Hemolytic assay. The hemolysis tests used are modifications of the method of Snyder and Bovarnick (11). 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 NaCl), and

the mixtures were centrifuged at $733 \times g$ for 10 min. The amount of released hemoglobin (in O.D. 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 Lowry method (8).

Electron microscopy. Samples for electron microscopy were fixed in glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate by the method of Schnaitman and Greenawalt (10).

Separation of adsorbed and unadsorbed rickettsiae in the hemolytic system. In many experiments, the rickettsia-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 $450 \times g$ 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 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 $733 \times g$ for 10 min. The released hemoglobin was read at 545 nm.

CO_2 evolution. Glutamate catabolism by typhus rickettsiae was determined by trapping $^{14}\text{CO}_2$ in Hyamine during incubation with ^{14}C -glutamate. Rickettsial suspensions (20% or 40%) in sucrose (0.218 M) phosphate (0.01 M, pH 7.0) buffer (SP) were incubated at 34 C in closed vials with uniformly labeled ^{14}C -glutamate (0.8 mM, 0.2 $\mu\text{Ci}/\text{ml}$). The total volume of the system was 1 ml; the $^{14}\text{CO}_2$ was released and trapped by the method of Fox (6).

Glutamate starvation. To deplete the rickettsiae of endogenous glutamate, the rickettsial suspension (80% or 100%) was incubated at 36 C for 30 min in SP buffer. The metabolic and hemolytic activity of these cells could be restored by the addition of glutamate.

RESULTS

Effect of time and temperature on adsorption. As shown in Fig. 1, the rickettsiae were rapidly adsorbed to the erythrocytes so that there was little remaining hemolytic activity in

FIG. 1. Time and temperature course of adsorption of rickettsiae to erythrocytes. Rickettsiae (20% suspension) and erythrocytes (25% suspension) were incubated at the times and temperatures indicated. The unadsorbed rickettsiae (supernatant) fractions were prepared and assayed as described in the text (Δ , 0 C; $\bullet$, 15 C; $\circ$, 25 C; $\blacktriangledown$, 34 C). The unadsorbed activity has units of absorbance at 545 nm. The adsorbed rickettsia-red blood cell (sediment) fraction was prepared and assayed after a 34 C incubation period for the indicated times ($\square$, dashed line). The adsorbed activity is expressed as percentage of the total hemolytic activity before fractionation. The arrow indicates the level of spontaneous hemolysis, which was not subtracted in these experiments and approximates the real basal level for the unadsorbed activity.

the supernatant fraction. The extent of adsorption was determined by incubating the supernatant fraction, which contained unadsorbed, free rickettsiae, with additional red blood cells. The time required for complete adsorption at 34 C varied from 2 to 4 min. Complete adsorption at 25 C and 15 C required approximately 5 and 10 min, respectively. At 0 C, as previously shown (9), there was no measurable adsorption, and all hemolytic activity remained in the supernatant fraction. Hemolytic activity appeared in the sediment fraction concomitant with the loss of activity in the supernatant fraction (Fig. 1). At 34 C, approximately 4 min was required for complete adsorption as determined by appearance of hemolytic activity in the sedimented fraction.

Metabolic inhibition of hemolysis. Hemolysis could be inhibited by a variety of metabolic inhibitors as shown in Table 1. In these experiments, the rickettsiae were incubated for 150 min in the presence of red blood cells, and the

TABLE 1. *Effect of metabolic inhibitors*

Inhibitor	Hemolytic activity		CO ₂ evolution	
	Absorbance	Inhibition (%)	nmol/120 min	Inhibition (%)
None	1.42	0	65	0
NaF (10 mM)	0.05	96	75	0
NaF (1 mM)	0.87	39	73	0
KCN (10 mM)	0.05	96	6	91
KCN (1 mM)	0.05	96	15	77
NaN ₃ (10 mM)	0.05	96	3	96
NaN ₃ (1 mM)	0.22	85	4	94
NaAsO ₂ (10 mM)	0.05	96	3	96
NaAsO ₂ (1 mM)	0.05	96	3	96

indicated concentration of inhibitor. Since rickettsiae should be insensitive to glycolytic inhibitors (e.g., NaF) and erythrocytes should be insensitive to inhibitors of oxidative phosphorylation (e.g., KCN), these results suggested that hemolysis required both metabolically active rickettsiae and erythrocytes. The target of the inhibitors was established by determining the effect of preincubation of rickettsiae or erythrocytes with the inhibitors on hemolytic activity and the inhibition of CO₂ evolution from glutamate by the rickettsiae (Tables 1 and 2). The inhibitors of oxidative metabolism, i.e., cyanide, arsenite, and azide, inhibited both hemolysis and the metabolism of glutamate by rickettsiae. On the other hand, fluoride inhibited hemolysis without affecting the catabolism of glutamate. The preincubation patterns in Table 2 demonstrated that the inhibition by fluoride was enhanced by preincubation of the red blood cells with the inhibitor. Preincubation of the rickettsiae with fluoride, however, did not increase the inhibition beyond that obtained without preincubation. In a similar experiment, preincubation of the red blood cells with cyanide had no effect beyond that obtained in the absence of preincubation, whereas preincubation of the rickettsiae with cyanide augmented inhibition. The preincubation experiments showed that the effects of the inhibitors, although rapid, were not immediate and confirmed the CO₂ evolution evidence that cyanide was inhibiting the rickettsiae and fluoride the red blood cells.

The effect of the inhibitors was most pronounced when they were present during the early phases of hemolysis. As shown in Fig. 2, both cyanide (1 mM) and fluoride (10 mM) essentially completely inhibited hemolysis if present at zero time (or before). However, if hemolysis was allowed to continue for 30 min, at which time two-

TABLE 2. *Effect of preincubation with inhibitors^a*

Preincubation period (min)	Preincubated component hemolytic activity (% of uninhibited rate)			
	Rickettsiae		Erythrocytes	
	KCN	NaF	KCN	NaF
0	10	71	20	59
6	5	72	21	23
10	4	70	22	22
20	2	69	23	22

^a Rickettsial suspensions (20%) were preincubated at 0 C with 3.3 mM NaF or 3.3 mM KCN. Red blood cells suspensions (25%) were preincubated at 0 C with 1.6 mM NaF or 1.6 mM KCN. Different concentrations of inhibitors were used in the preincubation suspensions to obtain a 1 mM final concentration in the hemolytic system. At various times, two volumes of erythrocytes were added to the rickettsiae and the hemolytic activity was assayed. The 150-min uninhibited control values (100% in the table) for the four experiments were the following: rickettsiae plus NaF, 2.07; erythrocytes plus KCN, 2.08; erythrocytes plus NaF, 1.86; rickettsiae plus KCN, 2.90.

thirds of the hemoglobin had been released, the release of the remaining one-third during the next 120 min was subject to an inhibition of only 50%. When the inhibitors were added after 50 min, the resulting inhibition was only 20%. To confirm this effect, the inhibitors were added to the adsorbed rickettsia-red blood cell (sediment) fraction obtained after centrifugation of the hemolytic mixture after various periods of incubation (Table 3). The inhibitory effects of both NaF and KCN decreased as the period of incubation prior to centrifugation was lengthened.

Metabolic inhibition of adsorption of rickettsiae to sheep red blood cells. As shown above, an early event in hemolysis appeared to be most dependent on the metabolic state of the rickettsiae and erythrocytes. We attempted to determine if adsorption was one of these early, energy-dependent events by investigating whether poisoned rickettsiae could adsorb to normal red blood cells and whether normal rickettsiae could adsorb to poisoned erythrocytes.

Adsorption of normal rickettsiae to fluoride-poisoned and unpoisoned erythrocytes occurred equally well, as shown in Tables 4 and 5. In the presence of 10 mM fluoride, hemolysis was inhibited 94%. However, if the supernatant and sediment fractions from the low-speed centrifugation were washed free of fluoride, hemolytic activity was found associated with the adsorbed rickettsia-red blood cell fraction (Table 4). No

FIG. 2. Decreased sensitivity with time of the hemolytic system to inhibition by metabolic poisons. A 20% rickettsial suspension was incubated with 25% red blood cells at 34 C. At various times, NaF (10 mM final concentration), KCN (1 mM final concentration), or NaCl plus formaldehyde (to stop the reaction) was added to the samples, and the samples were incubated at 34 C for a total of 150 min to determine hemolytic activity. The percentage of inhibition is expressed as the amount of hemolysis that occurred after the inhibitors were added in relation to the uninhibited hemolysis that could have occurred during this interval.

TABLE 3. Effect of inhibitors on hemolysis in the adsorbed rickettsia-red blood cell complex

Time ^a (min)	Inhibition (%)	
	KCN	NaF
5	80, 88	35, 41
10	63, 64	18, 39
20	45, 42	9, 25
30	37, 37	12, 20

^a The hemolytic mixture was incubated for the indicated time at 34 C and then centrifuged. The sediment fractions were resuspended with or without the inhibitors (1 mM) and incubated to determine hemolytic activity. The percentage of inhibition is relative to the uninhibited control value for each time period. The results of two experiments are shown.

additional hemolytic activity was detected in the unadsorbed rickettsiae fraction (Table 5). As a control, when the fluoride-poisoned hemolytic system was incubated at 0 C, the hemolytic ac-

TABLE 4. Hemolytic activity in the adsorbed rickettsiae-erythrocyte fraction after removal of NaF

Incubation components ^a	Unfractionated hemolytic activity (absorbance at 545 nm)	Hemolytic activity in adsorbed rickettsiae (sediment) fraction (% of unfractionated, uninhibited activity)			
		Resuspended		Resuspended and washed twice	
		With NaF	Without NaF	With NaF	Without NaF
RBC + NaF		4	4	3	3
Rickettsiae + RBC	2.16	1	85	2	55
Rickettsiae + RBC + NaF	0.13	4	60	3	65

^a The hemolytic system was incubated at 34 C and the adsorbed rickettsiae-red blood cell (RBC) fraction prepared. This sediment fraction was resuspended with or without NaF (10 mM) as indicated, centrifuged, and again resuspended.

tivity could be localized in the unadsorbed rickettsiae fraction after removal of fluoride. These results demonstrated that adsorption was independent of the energy metabolism of the red blood cell.

Similar experiments could not be performed using cyanide-poisoned rickettsiae since washing could not reverse the effect of cyanide as required to determine the localization of hemolytic activity. Therefore, instead of inhibiting energy production of rickettsiae with poisons, the rickettsiae were starved for glutamate to give an energy-depleted system. The effect of starvation could be easily reversed by the addition of glutamate. Table 6 showed that adsorption of energy-depleted rickettsiae to normal erythrocytes did not occur. Centrifugation of the mixture of starved rickettsiae and normal erythrocytes, in which hemolysis was less than 10% of the control level, yielded the usual fractions. However, in this case after restoration of energy metabolism by the addition of glutamate, the hemolytic activity could be recovered from the unadsorbed rickettsiae fraction but not from the adsorbed rickettsiae-red blood cell fraction.

These results were confirmed by electron microscopy examination of the sediment (adsorbed rickettsiae-red blood cell) fractions obtained from hemolytic systems incubated in the presence of

TABLE 5. *Adsorption of rickettsiae by NaF-inhibited erythrocytes*

Incubation		Hemolytic activity in unadsorbed rickettsiae (supernatant) fraction ^a (absorbance at 545 nm)
Inhibitor	Temperature (C)	
None	0	0.60
NaF	0	0.51
None	34	0.15
NaF	34	0.12

^a The supernatant fraction was prepared after a 10-min incubation period at the indicated temperature. The unadsorbed rickettsiae were washed three times by centrifuging at $12,000 \times g$ for 10 min and tested for hemolytic activity.

TABLE 6. *Glutamate requirement for adsorption of starved rickettsiae*

Incubation ^a		Hemolytic activity (% of combined, reconstituted activity)	
Additions	Temperature (C)	Unadsorbed fraction	Adsorbed fraction
None	34	93	7
MgCl ₂	34	89	11
Glutamate	34	28	72
Glutamate + Mg	34	19	81
Glutamate	0	92	8

^a A 100% rickettsial suspension, starved for glutamate, was diluted in SP to a 20% concentration and was incubated with 12.5% red blood cells for 10 min at the indicated temperature with the indicated additions to the media.

fluoride or cyanide. The procedure used was identical to that previously described (9) except that the sediment fractions were washed in presence of the inhibitor. As seen in Table 7, there were no rickettsiae adsorbed to the red blood cells in the cyanide-poisoned system. Analogous results were obtained, as previously reported (9), after incubation at 0 C. However, examination of the system with fluoride present showed rickettsiae associated with the erythrocytes (Table 7). As pointed out (9), the difficulties of quantitation of particles in thin section makes the numbers themselves insignificant. However, qualitatively, one finds rickettsiae associated with erythrocytes if, and only if, the rickettsiae are metabolically active. These results demon-

TABLE 7. *Electron microscopy of the sedimented fraction of the hemolytic system*

Incubation conditions ^a	No. rickettsiae/100 erythrocytes
No inhibitor, 0 C, unwashed	0
No inhibitor, 34 C washed	5
NaF, 34 C, unwashed	10
NaF, 34 C, washed	5
KCN, 34 C, unwashed	0
KCN, 34 C, washed	0

^a Rickettsiae and red blood cells were incubated and centrifuged at $455 \times g$ for 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.

strate that the metabolic activity of the rickettsia, but not the erythrocyte, must be intact for adsorption to occur. However, no lysis resulted from the adsorption of the rickettsia to the erythrocyte if the erythrocyte's metabolism was inhibited.

DISCUSSION

This study has emphasized that the hemolytic reaction is a complex one in which the metabolism of both components is involved. The dependence of the hemolytic system on the metabolic activity of the rickettsiae is demonstrated by the inhibitory effect of cyanide and glutamate starvation. The inability of fluoride to effect the oxidation of glutamate and yet inhibit hemolysis shows that the erythrocytes play an integral role in their own lysis. A possible parallel to the hemolytic system can be drawn from the work of Cohn et al. (5) who investigated the penetration of *R. tsutsugamushi* into mammalian cells in vitro. In this process, both the rickettsiae and the host cells must be metabolically active to obtain high levels of penetration.

Whereas the overall hemolytic process is dependent on the metabolism of both components, the first step in the process, adsorption, is dependent only upon the metabolism of the rickettsiae. That is, metabolically inhibited rickettsiae are not able to adsorb to normal erythrocytes, but metabolically normal rickettsiae can adsorb to metabolically inhibited erythrocytes. Although the adsorption step is apparently most sensitive to inhibition of rickettsial metabolism, it is not the only sensitive step. Adsorption is complete after 5 min at 34 C; however, inhibition by cyanide can be observed after the addition of KCN to the hemolytic mixture after as much as a 30-

min incubation. Because of the continuing, albeit decreasing, sensitivity of the hemolytic system to cyanide, rickettsial metabolism is probably necessary not only for adsorption but also for other processes that must occur to culminate in the lysis of the red blood cell. The sensitivity of the hemolytic system to fluoride inhibition indicates that the erythrocyte's metabolism is also important. Since the fluoride-poisoned red blood cells adsorb rickettsiae, but do not lyse, the fluoride-sensitive step(s) must be involved in processes other than the initial adsorption process. One part of the process that is dependent on the erythrocyte's metabolism must occur almost simultaneously with adsorption, since fluoride inhibits the hemolysis completely during the time of the adsorption process and then decreases. It is noteworthy that, although hemolysis in its late stages is insensitive to metabolic poisons, it can still be halted by lowering the temperature to 0 C.

The events following adsorption are certainly less clear. After adsorption hemolysis is dependent upon both temperature and the energy state of the erythrocytes and rickettsiae. The adsorbed rickettsiae could release a lysin, the release of which is dependent on the metabolism of the rickettsia, and its action is dependent on the metabolic activity of the erythrocyte. Alternatively, the rickettsia may be attempting to parasitize the erythrocyte and, analogous to the observation of Cohn et al. (5), require the metabolic activity of both host and parasite. Electron microscopy shows only a few instances where rickettsiae are present inside the red blood cell. The few internal rickettsiae observed did not appear to be in vacuoles. However, an attempt to invade the erythrocyte cannot be eliminated by the failure to observe the intracellular parasite, since the attempt could be abortive, resulting in lysis but no invasion. These studies have laid a foundation for the further investigation of the complex adsorption and hemolytic process.

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LITERATURE CITED

1. Bovarnick, M. R., and E. G. Allen. 1954. Reversible inactivation of typhus rickettsia. I. Inactivation by freezing. *J. Gen. Physiol.* **38**:169-179.
2. Bovarnick, M. R., and E. G. Allen. 1957. Reversible inactivation of the toxicity and hemolytic activity of typhus rickettsia by starvation. *J. Bacteriol.* **74**:637-645.
3. Bovarnick, M. R., J. C. Miller, and J. C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. *J. Bacteriol.* **59**:509-522.
4. Bovarnick, M. R., and J. C. Snyder. 1949. Respiration of typhus rickettsiae. *J. Exp. Med.* **89**:561-565.
5. Cohn, Z. A., F. M. Bozeman, J. M. Campbell, J. W. Humphries, and T. K. Sawyer. 1959. Study on growth of rickettsiae. V. Penetration of *Rickettsia tsutsugamushi* into mammalian cells *in vitro*. *J. Exp. Med.* **109**:271-292.
6. Fox, R. M. 1971. A simple incubation flask for $^{14}\text{CO}_2$ collection. *Anal. Biochem.* **41**:578-589.
7. Gimenez, D. F. 1964. Staining rickettsiae in yolk sac culture. *Stain Technol.* **39**:135-140.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin reagent. *J. Biol. Chem.* **193**:265-275.
9. Ramm, L. E., and H. H. Winkler. 1972. Rickettsial hemolysis: adsorption of rickettsiae to erythrocytes. *Infect. Immunity* **7**:93-99.
10. Schnaitman, C., and J. W. Greenawalt. 1968. Enzymatic properties of inner and outer membranes of rat liver mitochondria. *J. Cell Biol.* **38**:158-175.
11. Snyder, J. C., M. R. Bovarnick, J. C. Miller, and R. S. Chang. 1954. Observations on the hemolytic properties of typhus rickettsiae. *J. Bacteriol.* **67**:724-730.
12. Wisseman, C. L., Jr., E. B. Jackson, F. E. Hahn, A. C. Ley, and J. E. Smadel. 1951. Metabolic studies of rickettsiae. I. The effects of antimicrobial substances and enzyme inhibitors on the oxidation of glutamate by purified rickettsiae. *J. Immunol.* **67**:123-136.