OBSERVATIONS ON THE HEMOLYTIC PROPERTIES OF TYPHUS RICKETTSIAE[1]

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In 1948 Clarke and Fox reported that crude suspensions of typhus infected yolk sacs caused the lysis of erythrocytes of several animal species *in vitro*. When it was learned that typhus rickettsiae could be purified by differential centrifugation and treatment with celite in such a way as to reveal their independent metabolic activity (Bovarnick and Snyder, 1949) and to preserve their infectivity for cotton rats (Bovarnick *et al.*, 1950), it seemed of interest to determine whether the purified suspensions would still retain the hemolytic properties of the crude tissue suspensions. Preliminary tests showed that this was the case, and further experiments with purified rickettsial suspensions have made it possible to detect factors affecting the reaction and to develop a rapid, sensitive hemolysin test. The method as evolved during several years in two different laboratories is based on a 2.5 hour incubation of rickettsiae and red blood cells, followed by colorimetric determination of the hemoglobin released. For convenience this method is referred to subsequently as the "short hemolysin test" to distinguish it from the overnight procedure of Clarke and Fox in which the end point was determined by inspection.

This paper describes our standard procedure for the short hemolysin test and its application to quantitation of rickettsial suspensions under various conditions. Experiments on factors affecting the hemolytic activity of typhus rickettsiae are reported.

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MATERIALS AND METHODS

Diluents. The diluent for the rickettsiae and red cells in the standard short hemolysin test was sucrose PG (sucrose, 0.218 M; KH_2PO_4, 0.00376 M; K_2HPO_4, 0.0071 M; potassium glutamate, 0.0049 M; pH, 7.0; sterilized by autoclaving) (Bovarnick *et al.*, 1950) to which was added, after autoclaving, 10 ml of 0.1 M $MgCl_2$ per 100 ml. This was designated as sucrose PG + Mg.

In certain experiments solution NK 7 G was used for washing and resuspending the red blood cells. It contained KCl, 0.042 M; NaCl, 0.089 M; Na_2HPO_4, 0.0079 M; KH_2PO_4, 0.004 M; potassium glutamate, 0.005 M; pH 7.0. After autoclaving this solution, the following supplements were added per 100 ml: 0.4 ml of 50 per cent glucose and 8 ml of a solution composed of $MgCl_2$, 0.095 M; $CaCl_2$, 0.0025 M; and $MnCl_2$, 0.0025 M.

Rickettsiae. The Breinl strain of epidemic typhus, the Wilmington strain of murine typhus, and the human avirulent Madrid E strain (Gallardo and Fox, 1948) were maintained by propagation in embryonated eggs (Cox, 1941) for the various experiments as indicated below. The infected yolk sacs were homogenized in a Waring blendor, in the proportion, 50 g yolk sac for each 50 ml sucrose PG and shell frozen in a dry ice-alcohol bath for storage at −72 C. Suspensions of "once washed" rickettsiae (Bovarnick *et al.*, 1950) were stored in the same manner.

Toxicity for mice. The toxicity of various rickettsial suspensions was determined by inoculation of 0.25 ml of each dilution into the tail veins of 12 to 18 g mice, using 4 mice for each dilution. The LD_{50} was calculated by the method of Reed and Muench (1938) on the basis of survivors at 18 hours.

Erythrocytes. Sheep blood was drawn aseptically into an equal volume of sterile modified Alsever's

solution (sodium citrate, 0.027 M; sodium chloride, 0.072 M; glucose, 0.114 M; and citric acid, 0.0038 M) and stored in small screw cap bottles at +2 to 4 C. Sheep blood prepared in this way can be used for at least 4 weeks. On the day of the hemolysin test, the blood was centrifuged, and the supernatant plasma and buffy coat were discarded; the red cells were washed by centrifugation at low speed first with several volumes of sucrose PG, then twice more with sucrose PG + Mg, and finally suspended to a concentration of 25 per cent in the latter diluent.

Procedure of the short hemolysin test. Serial half-log dilutions of the rickettsial suspensions were made in sucrose PG + Mg; 0.2 ml portions of each dilution were placed in Kahn tubes, and 0.4 ml portions of the 25 per cent suspension of sheep erythrocytes were added to each tube. A control containing diluent in place of rickettsiae was set up at the same time. If the rickettsial suspension was turbid, as was the case with crude yolk sac suspensions, the hemolytic property of one set of dilutions was destroyed by heating at 56 C for 30 minutes before addition of the red cells to allow correction for the turbidity in the subsequent colorimetric measurements. Rickettsial dilutions and mixtures of red cells and rickettsiae were kept at 0 C until all suspensions to be tested at any one time had been prepared. The tubes were incubated then at 34 C for 2.5 hours. At the end of this time the hemolytic reaction was arrested by the addition to each tube of 2.0 ml of 0.85 per cent NaCl solution containing 0.2 ml formalin per 100 ml. The contents of each tube were well mixed, and the tubes were centrifuged at 2,000 rpm for 15 minutes. The optical density due to the hemoglobin released by the action of the rickettsiae was determined at wavelength 545 mμ in a Coleman Junior spectrophotometer. The sedimented intact red cells at the bottom of each tube were well below the light beam and thus did not interfere with the reading. For nonturbid suspensions, the tube containing diluent and red cells was used as the blank; for turbid suspensions, the corresponding heated dilution was used.

The end point was determined by plotting the optical density readings against the appropriate concentration of the rickettsiae. The concentration of the initial suspension was considered to be 1.0. The concentration of rickettsiae required to produce an optical density of 0.3 was read from the curve. (This was arbitrarily chosen as the end point because in this range a direct proportionality had been observed between concentration of rickettsiae and optical density.) The values thus obtained were of the same order of magnitude as the toxic LD_{50} for mice.

Example. Serial half-log dilutions of once-washed Breinl strain typhus rickettsiae, pool A, were tested as above. The readings were:

Dilution	*Rickettsial concentration*	*Optical density*
1/10	0.100	0.97
1/31.6	0.032	0.37
1/100	0.010	0.14

These values are plotted in figure 1 from which the concentration of rickettsiae required to give an optical density of 0.3 is found to be 0.024 or a dilution of the initial suspension of 1/42. This value is referred to hereafter as the hemolysin end point (HE). Pool A was shown to have a toxic LD_{50} for white mice at a dilution of 1/35.

Because of the short time required for the assay (2.5 hours at 34 C) it was not necessary to observe sterile precautions. The high concentration of red cells used in the above procedure was found essential to insure reasonable sensitivity since with lower concentrations the amount of hemolysis is very low. With the concentration of

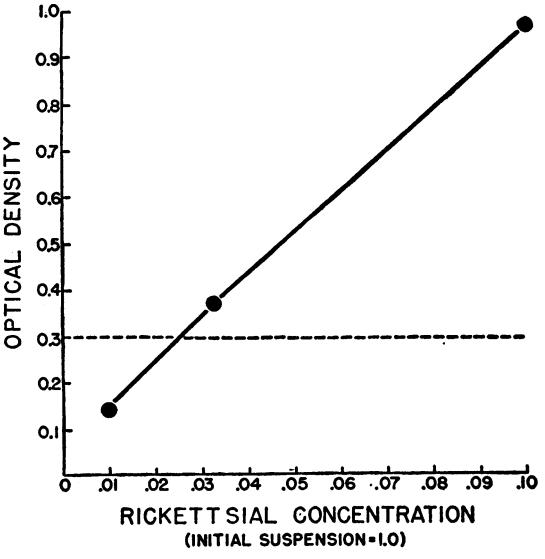

Figure 1. Example to illustrate determination of hemolysin end point of once-washed epidemic typhus rickettsiae, pool A.

rickettsiae normally used, the degree of hemolysis was small although it was possible to get complete hemolysis if a sufficiently high concentration of rickettsiae was present (approximately two hundred times that required for the chosen end point).

RESULTS

Reproducibility of hemolysin end points. The hemolysin end points for 5 different ampules of a single pool of once-washed Breinl rickettsiae were determined in separate standard short hemolysin tests performed on different days. The hemolysin end point values were 0.037, 0.040, 0.036, 0.032, and 0.036; the mean value was 0.036; and the standard deviation, 0.0029. A similar comparison was made with a pool of once-washed Madrid E strain; the hemolysin end point values were 0.048, 0.050, 0.050, and 0.056; the mean was 0.051; and the standard deviation was 0.003. These tests were run with erythrocytes from the same sheep, and the chance variations under these circumstances amount to less than 10 per cent. When erythrocytes from different animals were used, there was somewhat more variability.

Sensitivity. With the Madrid E strain the rickettsial end point was two to threefold higher with rabbit than with sheep red cells. It was usually more convenient to use sheep red cells because they are available in larger quantities in most laboratories.

The sensitivity with sheep erythrocytes can be increased by substitution of solution NK 7 G for sucrose PG. In this case the red cells were washed and suspended in NK 7 G containing magnesium, manganese, calcium, and glucose, and the rickettsiae are diluted in NK 7 G without metals. For assaying purified rickettsiae it was advisable to add normal yolk sac that had been heated for thirty minutes at 56 C in a final concentration of 0.5 per cent to the NK 7 G solution. This approximately doubles the titer with the Madrid E strain but makes a more cumbersome procedure than the standard short hemolysin test. The effects of various metals and of heated normal yolk sac on the hemolysin end points are shown in table 1.

Comparison of hemolysin end points and toxic LD_{50}. Titration of concentrated suspensions of typhus rickettsiae by intravenous inoculation of white mice is a very reliable and reproducible

TABLE 1

*Effect of various metals on the extent of lysis of sheep erythrocytes by typhus rickettsiae**

SOLUTION	SUBSTANCES ADDED				
	None	Mg†	Mg, Ca†	Mg, Ca, Mn†	Normal yolk sac‡
NK 7 G	11§	68	76	84	125
Sucrose PG	28	65		57	69

* The rickettsiae in the above experiment were Madrid E strain and had been purified as described by Bovarnick and Miller (1950).

† The final concentration of metals when present was: Mg, 4.1×10^{-3} M; Ca, 1.1×10^{-4} M; Mn, 1.1×10^{-4} M.

‡ For the NK 7 G solution, the normal yolk sac was added to the medium containing Mg, Ca, and Mn; for the sucrose solution, it was added to the mixture containing only Mg.

§ The figures represent the denominator of the dilution required to produce an optical density of 0.3.

TABLE 2

Comparison of the toxic LD_{50} for white mice and the hemolysin end point (HE) of various pools of typhus rickettsiae

RICKETTSIAL STRAIN	PREPARATION	NO. OF COMPARISONS	Ratio: LD_{50}/HE*	
			Mean	S.D.
Breinl epidemic	crude yolk sac	40	1.03	0.51
Breinl epidemic	once washed	4	1.26	0.28
Madrid E	crude yolk sac	5	0.96	0.22
Madrid E	once washed†	3	0.56	0.20
Madrid E	once washed	12	1.68	0.42
Wilmington murine	crude yolk sac	16	0.51	0.45
Wilmington murine	crude yolk sac	3	0.27	0.11

* The LD_{50} and the HE values used in calculating the ratios were expressed as concentration of rickettsiae referred to the initial suspension as 1.0. If, as in other tables, denominators of end point dilutions are used in the calculations, the values of ratios are the reciprocals of those indicated in this column.

† This pool was frozen and thawed twice after washing.

procedure with sharp end points (references cited by Neva and Snyder, 1952). Comparisons of the hemolysin end points and the toxic LD_{50} values were carried out by performing the two tests simultaneously each time an ampule of rickettsiae was thawed out for use. Table 2 shows the results of 83 comparisons with seven different rickettsial preparations.

Since a difference of one mouse in the toxicity titrations, as performed in these experiments, involves an appreciable change in the LD_{50} value, the variability recorded in table 2 is approximately what would be anticipated. The ratios vary somewhat from strain to strain and from one pool to another of the same strain. The differences observed between crude yolk sac preparations, washed preparations, and frozen washed preparations may reflect partial injury, resulting in selective destruction of one or the other property (compare table 3). The physiologic state of the rickettsiae at the moment of harvest of the yolk sacs, e.g., the proportion of fully infective to inactive cells, may be another aspect of importance. Finally, the strains themselves may differ in relative toxic and hemolytic activities.

TABLE 3

Comparison between loss of toxicity for mice and hemolytic activity of rickettsiae on incubation for 16 hours at 34 C

EXPERIMENT*	SOLUTION	HEMOLYTIC ACTIVITY†			TOXICITY FOR MICE†		
		Initial titer	Final titer	Per cent change	Initial titer	Final titer	Per cent change
1	A	5.8	3.3	−44	5.5	<0.7	>−88
	B	5.5	4.4	−20	5.5	1.4	−74
2	A	6.4	6.4	0	5.5	1.7	−69
	B	6.4	6.8	+6	5.5	2.8	−49

* In these experiments washed rickettsiae were suspended in a concentration equivalent to 10 per cent original infected yolk sac, and the suspensions were assayed before and after incubation. The solutions used were: A, the sucrose medium described by Bovarnick, Allen, and Pagan (1953) or B, the same medium with the addition of diphosphopyridine nucleotide.

† The figures represent the denominators of the dilutions required to produce an optical density of 0.3 in the hemolysin assay, or death of 50 per cent of the mice in the mouse toxin assay.

In view of the degree of correlation found between these two quite different phenomena, it is evident that the *in vitro* test can be used under ordinary circumstances in place of the animal test for the assay of crude or once washed typhus rickettsiae.

The parallelism between toxicity for mice and hemolytic activity did not always hold when rickettsiae were subjected to conditions leading to partial inactivation. When rickettsiae were incubated for 16 hours at 34 C, the loss in toxicity for mice appeared to be appreciably more rapid than the loss in hemolytic activity, as can be seen in table 3.

Factors affecting the hemolysis by typhus rickettsiae. In working out the procedure for the standard short hemolysin test, the influence of various conditions was studied. It was found that the optimum pH was about 7, and that changes in the time of incubation from 2 to 6 hours had very little effect on the end point. Glucose had no effect on the end point but was included in the NK 7 G medium to decrease the spontaneous hemolysis of the red cells.

When purified rickettsiae were used, it was possible to show that both magnesium (see table 1) and glutamate (see table 4) were required for the lysis of red cells by rickettsiae. When the salt medium was used, calcium and manganese

TABLE 4

*Effect of glutamate on the lysis of red cells by typhus rickettsiae**

TYPE OF RED CELLS	TIME OF INCUBATION	NO GLUTAMATE PRESENT	GLUTAMATE† ADDED AT	
			0 time	2 hours
	hr			
Sheep	2	—	71	18
	4	20	83	71
Rabbit	3	10	133	—

The figures represent the denominator of the dilution required to produce an optical density of 0.3.

* The above experiments were carried out as usual, except that glutamate was omitted from the sucrose PG+Mg solution used in washing the red cells and in diluting the rickettsiae.

† Glutamate, 0.02 ml of 0.15 M, was added to the red cell-rickettsiae mixtures either before incubation or after two hours' incubation at 34 C, as indicated.

had a small additional effect in increasing the degree of hemolysis. The magnitude of the effect of the added metals and glutamate varied with the purity of the rickettsiae, the greater the concentration of yolk sac, the less apparent the requirement for any of these factors, presumably due to their presence in the yolk sac tissue.

Using rabbit erythrocytes it was noticed that one other substance, namely adenosine triphosphate (ATP), had a marked effect on the degree of hemolysis. A concentration of washed rickettsiae, sufficient to produce a low degree of hemolysis corresponding to the end point chosen in the above described procedure under the usual conditions, brought about complete hemolysis of the rabbit cells when adenosine triphosphate (0.0004 M to 0.005 M) was added to the reaction mixture. This phenomenon has been found to be related only indirectly to the rickettsiae. Rabbit erythrocytes alone were unaffected by the presence of adenosine triphosphate. When a small amount of a water hemolysate of rabbit erythrocytes as well as adenosine triphosphate was added to undamaged rabbit cells, complete hemolysis took place after one to two hours at 34 C. The amount of hemolysate required was of the same order of magnitude as that produced by rickettsiae in the absence of adenosine triphosphate (see table 5).

TABLE 5

Influence of adenosine triphosphate (ATP) on the lysis of rabbit erythrocytes

RICKETTSIAE OR HEMOLYSATE ADDED	CONCENTRATION OF ATP	
	0	0.0004 M
	Optical density reading	
None	0.0	0.0
Rickettsiae* 1/2,000	0.04	0.05
Rickettsiae* 1/600	0.13	0.8
Rickettsiae* 1/200	0.28	1.3
Rickettsiae* 1/60	0.78	1.3
Rabbit cell† hemolysate 1/8	0.08‡	0.08
Rabbit cell† hemolysate 1/4	0.17‡	1.4
Rabbit cell† hemolysate 1/2	0.34‡	1.4

In the above experiment, each tube contained 0.3 ml 8 per cent rabbit cells. The other constituents were added in the following amounts, where indicated: rickettsiae, 0.2 ml; ATP, 0.0024 M, 0.1 ml; hemolysate, 0.2 ml; sucrose PG+Mg to bring the total volume to 0.6 ml. The diluent used throughout was sucrose PG+Mg. The remainder of the experiment was carried out as usual.

A lower concentration than usual of rabbit cells was used in these experiments so that optical density readings on complete hemolysis could be obtained. An optical density reading of 1.4 corresponds to complete hemolysis in this test.

* The rickettsiae were a washed preparation of the Madrid E strain.

† The hemolysate was prepared by addition of 15 ml water to 1 ml packed, washed rabbit cells. After 3 to 4 minutes, 3.7 ml 42.5 per cent sucrose was added to restore isotonicity.

‡ These readings represent the color due to the added hemolysate.

Metabolic inhibitors. Several metabolic inhibitors were tested for their effect on the lysis of sheep red cells by typhus rickettsiae. Table 6 shows that cyanide, azide, fluoride, and iodoacetate reduce the hemolysis under the conditions of the test.

TABLE 6

Effect of inhibitors on the lysis of sheep erythrocytes by typhus rickettsiae

INHIBITOR‡	SOLUTION			
	NK 7 G*		Sucrose PG†	
	Expt 1	Expt 2	Expt 1	Expt 2
	Per cent Inhibition			
NaCN, 1×10^{-4} M	94	93	64	65
NaN_3, 1×10^{-3} M	61	55	24	
2×10^{-3} M				57
NaF, 4×10^{-3} M	89	89	93	93
Iodoacetate, 10^{-3} M	68	62	41	
2×10^{-3} M				73
Malonate, 10^{-2} M	7		0	

* The NK 7 G solution contained also Mg, Ca, and Mn, and heated normal yolk sac in the concentration described in the test.

† Mg was added to the sucrose PG solution.

‡ The inhibitors were added to the mixture of red cells and rickettsiae at 0 C immediately before transferring the tubes to 34 C.

Effects of antibiotics on the hemolysin end point. Several tests were carried out to determine the effect of aureomycin, chloramphenicol, and terramycin on the hemolysin end points of representative pools of the Breinl, Madrid E, and Wilmington strains. In each test the rickettsiae were washed once before the antibiotics were added. The general plan was exposure of the rickettsial suspension at 34 C for varying intervals up to 4 hours, to different concentrations of antibiotics up to 300 μg per ml in sucrose PG solution, with suitable controls. The red blood cells were added then, and the tests were run as described above. In two experiments the attempt was made to eliminate the antibiotic from the rickettsial suspension by two cycles of high speed centrifugation at 2 to 4 C before addition of the red cells. The results of a typical experiment are shown in table 7.

The hemolysin end points were reduced sharply by exposure of rickettsiae to aureomycin and terramycin; chloramphenicol had no effect under the conditions of these tests. Furthermore, the hemolysin end point values were unchanged by two cycles of washing of the rickettsiae following their contact with the antibiotics.

TABLE 7

In vitro effect of antibiotics on hemolysin end points (HE) of epidemic typhus rickettsiae

ANTIBIOTIC	CONCENTRATION	HEMOLYSIN END POINTS AFTER SPECIFIED TIME OF CONTACT AT 34 C*			
		Zero	1 hr	4 hr	4 hr then washed twice
	μg/ml				
None	0	49	59	51; 51‡	38‡
Aureomycin	100†	36	32	25	
	316	22	<3	<3	
	316			<2‡	<2‡
Terramycin	100†	46	48	45	
	316	56	45	<3	
	316			20‡	8‡
Chloramphenicol	100	44	50	38	
	316	36	44	44	

* Figures are denominators of dilutions required for optical density of 0.3.

† Concentrations less than 100 μg/ml had no appreciable effect on the HE values.

‡ Madrid E strain; all other values shown refer to the Breinl epidemic strain.

DISCUSSION

The short hemolysin test which has been described in this paper is a convenient assay procedure for typhus rickettsiae in relatively concentrated suspensions. During its application in two different laboratories over several years, it has been shown to have the same order of sensitivity and reproducibility as the titration of toxicity by the intravenous inoculation of white mice. If a laboratory has an abundant supply of sheep or rabbit red blood cells, the test is less expensive to perform than the toxicity titration in mice. The short procedure herein described has obvious advantages over the original procedure used by Clarke and Fox (1948). It is apparent, however, from the variety of factors which affect the degree of hemolysis brought about by a given concentration of rickettsiae that the hemolysin test is much more subject to influence by other substances present in the rickettsial suspension than is the mouse toxin assay.

The phenomenon of *in vitro* lysis appears to be a process which requires magnesium and glutamate and possibly calcium and manganese as well. The effect of normal yolk sac in increasing the hemolysis by washed rickettsiae even in the presence of glutamate and magnesium presumably is due to its stabilizing effect on the rickettsiae. It is of interest to note that glutamate can still promote hemolysis after the red cells and rickettsiae have been incubated together for two hours alone (see table 4), and thus it would appear that glutamate must be required for some reactions which precede or take part in the hemolysis. It may be pertinent in this respect that glutamate is the only known substrate which is metabolized rapidly by rickettsiae.

The inhibition of hemolysis by cyanide, azide, and iodoacetate parallels closely their inhibition of the oxidation of glutamate by typhus rickettsiae (Wisseman *et al.*, 1951; also, unpublished observations of the authors). On the other hand, the hemolysin reaction is very much more sensitive to fluoride than is the oxidation of glutamate. The best known example of a reaction highly sensitive to fluoride is that of the enolase of glycolysis. Rickettsiae show no evidence of glycolysis, but red cells do. Whether this indicates that some reaction of the red cells also contributes

to their own lysis remains to be determined. In agreement with this hypothesis, however, is the fact that the lysis of rabbit cells brought about by rabbit hemolysate and adenosine triphosphate also is inhibited strongly by fluoride, though not affected by cyanide (unpublished experiments). In any case, the significant inhibition of the rickettsial hemolysis by a variety of metabolic inhibitors indicates that at least one and probably several enzymatic reactions are involved in this hemolysis. A similar conclusion was reached by Gardner and Morgan (1952) in a study of the mumps hemolysin although the effective inhibitors differ markedly in the two reactions.

The effects of aureomycin, terramycin, and choloramphenicol on the hemolysin are quite similar to their effects on the oxidation of glutamate by rickettsiae (Karp and Snyder, 1952). The unsuccessful attempts to restore hemolytic activity of rickettsiae after exposure to terramycin and aureomycin by centrifugation and resuspension suggest that these antibiotics become firmly attached to the rickettsial cells, or that they exert an irreversible effect on the enzyme systems involved in the hemolysin reaction.

SUMMARY

A rapid test for the demonstration of hemolytic properties of typhus rickettsiae is described. The test is of the same order of sensitivity and reproducibility as the titration of toxicity by intravenous inoculation of white mice. The hemolysin reaction appears to require glutamate and magnesium and possibly calcium and manganese as well. Metabolic inhibitors reduce the hemolytic activity of rickettsiae. Aureomycin and terramycin reduce the hemolytic activity of rickettsiae, whereas chloramphenicol does not.

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