

THE PHENOMENON OF IN VITRO HEMOLYSIS PRODUCED BY
THE RICKETTSIAE OF TYPHUS FEVER, WITH A NOTE ON
THE MECHANISM OF RICKETTSIAL TOXICITY IN MICE

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The rapid death of mice inoculated with yolk sac suspensions richly infected with the rickettsiae of murine typhus was first reported in 1940 (1). Shortly thereafter it was shown that similar suspensions of rickettsiae of epidemic typhus possess the same toxic property (2); and a serum neutralization technique based on this property was developed (3), which was highly specific in differentiating antibody of murine or of epidemic typhus origin (4). A similar toxic property has been demonstrated in yolk sac suspensions of *R. orientalis* of a single strain (Gilliam) only (5). The nature of the "toxin" has not been established, although it is apparently inseparable from the living rickettsial bodies (2). The mechanism by which death is produced in the mice has not been investigated.

Postmortem examination of mice dying after inoculation with suspensions of rickettsia-infected yolk sac suggested that blood clotting *in vivo* might play a rôle in the mechanism of death (6). In following this lead, a study was made of the effect of normal and infected yolk sac suspensions on heparinized whole blood of mice, guinea pigs, and rabbits. Contrary to what had been anticipated, it was observed that clotting occurred much more slowly in mouse blood mixed with infected yolk sac than it did in mouse blood mixed with normal yolk sac or with a combination of infected yolk sac and specific immune serum. Although this inhibition of clotting proved to be a reproducible phenomenon in the case of mouse blood, such was not the case with blood from the guinea pig and the rabbit. Instead, a different and equally interesting phenomenon was observed; *i.e.*, *in vitro* hemolysis of the heparinized rabbit blood by yolk sac preparations infected with typhus rickettsiae.

Normal yolk sac prepared in the same manner as the infected lots in no case showed hemolytic activity. The hemolytic principle of infected yolk sacs was recoverable from the sediment after centrifugation at speeds demonstrated to throw down most of the rickettsiae (4,000 to 5,000 R.P.M. for an hour). Finally, the hemolytic activity of these preparations was observed to be inhibited by the presence of immune serum. Further studies were indicated to determine the optimal conditions for the demonstration of hemolytic activity, with a view to

the investigation of the mechanism of specific hemolysis and of possible methods of application.

This report describes the hemolytic property of typhus rickettsiae, with observations on the mechanism of hemolysis and the utilization of this property in the development of a new serologic technique. Also described are brief *in vivo* experiments to determine the possible relation of the hemolytic property of rickettsiae to their toxic activity in mice.

Methods

Since description of the development of a technique for the demonstration *in vitro* of hemolysis caused by rickettsiae and of its inhibition with immune serum, constitutes an integral part of this paper, only certain basic procedures need be mentioned in this section.

The principal rickettsial strains employed were the Breinl (epidemic typhus) and the Wilmington (murine typhus), both of which had been maintained for a long but unrecorded series of passages in chick embryo yolk sac. Supplementary observations were also made with the South American strain (epidemic typhus) and the Gear strain (murine typhus), which were in relatively early passage (8th to 10th) in eggs. Yolk sac pools were prepared from eggs infected with rickettsiae by inoculation into the yolk sac (7) and harvested after 6 to 7 days' incubation at 35°C. The pooled yolk sacs were homogenized in a Waring blender as 20 per cent suspensions by weight in nutrient broth and were stored in sealed glass ampoules in a dry-ice chest until used. Only pools shown to be sterile by test cultures on nutrient agar and in glucose and thioglycollate broth, were employed. All dilutions of yolk sac indicated in the paper were made from the 20 per cent suspensions in nutrient broth, which were considered the original undiluted material.

Blood for the substrate for the tests was collected chiefly from the rabbit, by cardiac puncture. Heparin, to give a concentration in the blood of 1:10,000 to 1:20,000, or sodium citrate, to give a concentration of 0.1 per cent, was used when clotting was to be avoided. In addition to rabbits, the animals employed as a source of either blood or serum or for the demonstration of toxicity or infectivity of yolk sac pools were sheep, guinea pigs, eastern cotton rats (*Sigmodon hispidus hispidus*), and albino Swiss mice.

Pyrex tubes measuring 10 to 12 mm. by 70 mm. proved convenient for both hemolytic titration and serum inhibition studies. For incubation at any temperature above that of the room an ordinary bacteriological incubator adjusted to the desired temperature was used, unless otherwise stated. In a few cases where a short incubation period was employed, a water bath was used. Hemolysis was read on a scale ranging in degree from 4+ (very deep color all through the supernatant above the settled cells) through 3+, 2+, 1+, trace (tr.), and faint trace (ft. tr.), in the last of which only a minimum of hemoglobin had diffused into that supernatant immediately overlying the cells. Where end-points of hemolysis have been indicated they correspond to the dilution giving a reading of "tr."

Determinations of the toxicity of infected yolk sac were made by inoculating 0.25 ml. of serial twofold dilutions of suspended yolk sac into the tail veins of mice, and observing the number of mice dead after 18 to 24 hours (2). Lethality for cotton rats was demonstrated by inoculating similar yolk sac dilutions, also in 0.25 ml. volume, intracardially; the rats were then observed for a period of 10 days, during which time the number of deaths occurring was recorded (8). Infectivity determinations were made by inoculating 0.25 ml. volumes of serial fourfold dilutions of yolk sac intraperitoneally into cotton rats, which were challenged

intracardially 4 weeks later with a dose of between 2 and 4 LD₅₀. Rats surviving such challenge were considered to have been immunized by the original inoculum. Toxic, lethal, and infective titers were calculated by the 50 per cent end-point method (9).

EXPERIMENTAL

Factors Conditioning the Demonstration of Specific Hemolytic Activity

Volume Relationships.—The relative proportion of rickettsia-infected yolk sac dilution to red cell substrate does not appear to be of critical importance in preparations to be used for the observation of hemolytic activity. Identical hemolytic titers were obtained with either 2 parts of yolk sac to 5 parts of substrate or with equal volumes of each. The use of 5 parts of yolk sac to 2 parts of substrate resulted in a twofold reduction in titer. The volumes adopted for use were 0.2 ml. of yolk sac dilution and 0.5 ml. of substrate. Increase in these volumes up to 8 times without altering the proportions of the components had no effect on the titers.

Red Blood Cell Substrate.—The optimal composition of the red blood cell substrate was studied in considerable detail, and it is probable that the results obtained have some bearing on the mechanism of the hemolytic phenomenon, the nature of which remains obscure. The use of whole heparinized blood as the substrate, which was tried at first, proved unsatisfactory because the frequent occurrence of clotting in this material interfered with the observation of the degree of hemolysis. In an effort to overcome this difficulty, experiments were made with red cells plus saline and plasma or serum in varying proportions. The results of these experiments are summarized in Table I. Consistently, the maximum hemolytic titers have been observed when the cells constituted 25 per cent or more of the substrate, while the use of fewer cells resulted in proportionately lower titers. A corollary to this observation of the interdependence of red cell concentration and hemolytic titer is the fact that hemolysis of all the cells in the substrate has never been observed; decrease in the concentration of cells in an effort to obtain complete hemolysis has led instead to a decrease in degree of hemolysis. Thus, the concentration of red cells present in the substrate appears to be a critical factor in hemolysis.

The composition of the substrate, apart from the concentration of the red cells, seems to be of minor importance. Plasma was ruled out for routine use because of the frequent occurrence of clotting when it was employed. The substrate adopted was 25 per cent red blood cells, 25 per cent serum, and 50 per cent saline. This combination was decided upon because results of several experiments, such as Experiments 3 and 4 listed in Table I, suggested that it yields slightly higher and more consistent titers. Sodium citrate may be substituted for heparin as an anticoagulant for use in collecting the red cells. In some of our experiments we have employed as the substrate defibrinated blood diluted with an equal volume of saline. This has yielded satisfactory results at times, but cells prepared in such a way may undergo spontaneous hemolysis, due perhaps to physical injury to the cells during the process of defibrination.

The animal used as donor of the red blood cells employed in most of our studies has been the rabbit. In a few tests sheep cells were employed with essentially similar results; but the lack of a source for aseptic bleeding in the case of this animal caused us to discontinue its use, since its red cells offered no apparent advantage over those of the rabbit. As a matter of interest, the cells of other animals were investigated for their susceptibility to the hemolytic action of typhus rickettsiae. Erythrocytes of the mouse, cotton rat, and guinea pig all proved completely resistant to hemolysis when suspended in dilutions of either their own or rabbit serum.

The use of sera from species other than the rabbit as a component of the substrate was

explored. This was of practical importance since such sera were to be employed in studying the inhibition of the hemolytic activity by specific immune sera. It was observed that fresh guinea pig or cotton rat serum occasionally caused some hemolysis of rabbit cells, but this was generally found to be reduced or eliminated by heating the serum at 56°C. for 1 hour. Such heated serum when present in relatively large amount (25 per cent of the red cell substrate) in general led to slight reduction in the hemolytic titer as compared with that obtained with rabbit serum; but this reduction was not of sufficient degree to invalidate its use in small amount for serological testing. It was further shown that similar heating of the rabbit serum

TABLE I
Relation between Hemolytic Titer of Infected Yolk Sac Pools and the Composition of the Red Cell Substrate

Rickettsia strains used to infect yolk sac*	Experiment No.	Composition of substrate Per cent of				Hemolytic titer (1:x)
		Plasma‡	Serum§	Saline	Red cells†	
Wilmington	1	25		50	25	128
		25		65	10	64
		25		70	5	32
	2	50		25	25	64
		25		50	25	64
		10		65	25	64
Breinl	3		75		25	32
			25	50	25	64
				75	25	64
				90	10	8
	4		25	50	25	64
				75	25	32

* Dilutions with saline.

† Plasma and red cells obtained from heparinized rabbit blood.

§ Serum obtained from rabbit blood drawn without anticoagulant.

|| Titer read as dilution of 20 per cent infected yolk sac in which a trace of hemolysis was observed.

component of the usual substrate did not affect the demonstration of the hemolytic activity of suspensions of infected yolk sac.

Temperature and Time of Incubation.—The hemolytic reaction was found to be favored by moderately warm temperatures. In a typical experiment with yolk sac infected with rickettsiae of epidemic typhus, hemolysis was negligible when the mixtures of yolk sac and substrate were kept for 24 hours at 4°C., whereas after the same period at room temperature or at 35°C. the hemolytic end-points were 1:128 and 1:256 respectively. From successive readings made after 2, 4, and 18 to 24 hours of incubation at 35°C., it was also evident that hemolysis was not instantaneous but was relatively slow moving and progressed even after 4 hours' incubation, since the end-points observed at the 4-hour interval were one or two dilutions lower (two to four times less) than those observed in the same titration series after

18 to 24 hours. The significance of these findings in relation to the study of the reaction will be discussed subsequently. A time period of 18 to 24 hours at 35°C. has been selected as giving the optimal hemolytic titers. It is, of course, necessary to avoid bacterial contamination of all reagents in the performance of a test requiring such a prolonged incubation at a warm temperature.

The Hemolytic Principle

Hemolytic activity has been demonstrated in yolk sac preparations of all the strains of murine and epidemic typhus rickettsiae tested. It has also been found in a liver suspension from cotton rats infected with the Gear strain of the murine typhus rickettsia and in fresh peritoneal washings from cotton rats infected with the Wilmington strain of this rickettsia. Titers obtained varied from pool to pool of infectious material, but the majority of yolk sac pools rich in rickettsiae showed end-points of from 1:32 to 1:128. Preparations of several strains of *R. orientalis* (the organism of tsutsugamushi disease), including the Gilliam strain, have never exhibited any hemolytic activity.

Stability of the Hemolytic Principle.—One of the early observations on the nature of the hemolytic principle was its marked instability. Exposure of active yolk sac suspensions to 0.5 per cent formalin, which renders rickettsiae non-infective, was also shown to destroy the hemolytic principle completely. The far less drastic treatment of exposure to moderate temperatures was found to be accompanied by partial or complete loss of activity. The results of the studies on the effect of various temperatures upon 20 per cent yolk sac suspensions, summarized in Table II, illustrate this thermolability. Progressive loss of activity was observed even over a few hours' time at room temperature or at 35°C. Material kept for 8 hours at room temperature and overnight at 4°C. showed almost a complete loss of activity. Storage at refrigerator temperature (4°C.) retarded this effect, although a fall in titer was observed after a 1- to 2-day period. Heating at 56°C., which destroys the infective nature of rickettsial preparations, also resulted in the complete loss of hemolytic activity.

The relation of the type of diluent employed to the stability of the hemolytic principle was investigated as a point of practical importance. Definitely higher titers were obtained when the serial dilutions of active yolk sac were made in skim milk or in 20 per cent normal yolk sac than when any other diluent was employed. Such diluents, however, have not been regularly employed owing to the impossibility of estimating end-points accurately in turbid materials. For routine use physiological saline has been adopted, since other clear materials tested, such as diluted serum or nutrient or glucose broth, yielded no better results.

High Speed Centrifugation.—Centrifugation studies were carried out with the double purpose of investigating the nature of the hemolytic principle and of removing extraneous yolk sac material, which possibly inhibited the reaction of

hemolysis and which certainly rendered more difficult the reading of hemolysis in low dilutions of yolk sac. A Pickels' angle-head centrifuge was employed to sediment the rickettsiae, and most of the work was carried out in the cold room to minimize losses due to the instability of the active material.

The results obtained (Table III) indicated that the hemolytic activity moved with the sedimentable material, the supernatant fluid being devoid of such activity. Concentration of the sediment by resuspension in a smaller volume of saline, gave rise to higher hemolytic titers. Such sedimented preparations, when resuspended to volume in saline and tested immediately, were relatively

TABLE II
Loss of Hemolytic Titer of Infected Yolk Sac Suspensions on Exposure to Various Temperatures Prior to Testing

Exposure of infected yolk sac*		Hemolytic titer† (1:x)	
Temperature	Time	Unexposed control	Exposed
°C.			
4	1 day	64	16
4	2 days	64	32
20-25	2 hrs.	64	32
20-25	4 "	64	8
20-25	8 " §	64	2
35	2 "	32	16
35	3 "	128	32
35	4 "	32	8
56	1 hr.	32	0

* Yolk sacs were infected with either Wilmington murine or Breinl epidemic strains of rickettsiae, and were exposed in 20 per cent concentration. At the end of the exposure period, dilutions were made in saline and added to the red cell substrate.

† Titers are indicated as in Table I.

§. After exposure, stored at 4°C. overnight prior to testing.

clear and made satisfactory preparations for the demonstration of hemolytic activity despite some loss in potency during processing. The preservation of such preparations for future tests was rendered impossible, however, by their complete loss of activity upon storage, in a saline suspension, even at -70°C . (in a dry-ice chest). The latter observation is an especially marked example of the instability of the hemolytic factor. In this case, the loss of activity was apparently due to the fact that the preparations were relatively free from protective yolk sac material. Diluted rabbit serum slightly retarded the loss of potency, while skim milk proved an excellent preservative over an 8-day storage period. No practical advantage was gained, however, by the use of the latter medium since the purpose of centrifugation, *i.e.* to clarify the preparation and facilitate reading of the degree of hemolysis, was thereby defeated.

The Quantitative Relationship of in Vitro Hemolytic Activity to in Vivo Rickettsial Effects.—From the evidence already presented, a close relationship is suggested between the hemolytic property of suspensions of infected yolk sac and the contained, living rickettsiae. The diluents, such as normal yolk sac suspen-

TABLE III
*Centrifugation Studies on Infected Yolk Sac Suspensions**

Experiment No.	Fraction	Suspending medium†	Duration of storage‡	Hemolytic titer (1:×)	
				Un-treated control	Treated
1	Sediment Supernatant	Saline	Used immediately	32	16
			“ “	32	0
2¶	Sediment	Saline to $\frac{1}{4}$ original volume	Used immediately	128	256
3¶	Sediment	Saline to $\frac{1}{4}$ original volume	Used immediately	32	128
4**	Sediment	Saline	48 hrs.	64	0
	Sediment	25 per cent rabbit serum	Used immediately	64	32
	“	25 per cent rabbit serum	48 hrs.	64	4
5**	“	25 per cent rabbit serum	8 days	64	Undiluted
	“	Skim milk	Used immediately	64	32
	“	“ “	48 hrs.	64	32
	“	“ “	8 days	64	32

* Yolk sac suspensions were infected with Breinl epidemic or Wilmington murine strains of rickettsiae.

† Unless otherwise indicated, sediments were resuspended to original volume.

‡ Storage at -70°C . (dry-ice chest).

|| Centrifugation once at 5,000 R.P.M. for 1 hour at room temperature.

¶ Centrifugation once at 5,000 R.P.M. for 1 hour in cold room.

** Centrifugation at 5,000 R.P.M. for 1 to $1\frac{1}{2}$ hours; resuspension of sediment in saline and centrifugation for 10 minutes at 1,000 R.P.M.; the supernate then centrifuged at 5,000 to 8,000 R.P.M. for 1 to $1\frac{1}{2}$ hours; all done in cold room.

sions and skim milk, known to be most effective in preserving the infectivity (and hence, presumably, the viability) of rickettsial preparations, also offered the best protection for the hemolytic activity. Like the factor toxic for mice (2) the “hemolysin” could not be separated from the rickettsiae by centrifugation,

and treatment such as with 0.5 per cent formol or exposure to 56°C. for an hour, which renders rickettsiae non-infective, also destroyed the "hemolysin."

Further evidence of the relation of the "hemolysin" to the rickettsiae present was obtained by the simultaneous determination of the hemolytic titer and the titer of such other manifestations of rickettsial presence as toxicity for mice and lethality and total infectivity for cotton rats under conditions known to result in a decrease or an increase in the level of hemolytic activity. The conditions employed were exposure for 3 hours to a temperature of 35°C. (*cf.* Table II) or concentration of the active principle by resuspending the sediment obtained by high speed centrifugation in one-fourth the original volume of fluid (*cf.* Table

TABLE IV
Comparative Observations on Hemolytic Activity, Toxicity for Mice, and Lethality and Total Infectivity for Cotton Rats, of Infected Yolk Sac Pools

Rickettsial strain	Treatment of yolk sac	End-point dilutions* (1:z)			
		Hemolytic	Toxic† (for mice)	Lethal§ (for rats)	Infective (for rats) (× 10 ⁶)
Wilmington murine	Fresh	128	22.3	42.5	1.3
	3 hrs. at 35°C.	32	5.3	12.9	0.45
	4 × concentrated	256	77.6	128.0	10.0
Breinl epidemic	Fresh	128	56.1	306.0	10.8
	3 hrs. at 35°C.	32	13.1	11.3	5.4
	4 × concentrated	512	181.0	512.0	22.0

* Starting material (20 per cent suspensions of yolk sac) considered as undiluted. Inoculum for rats and mice, 0.25 ml. volume. End-points other than hemolytic calculated by 50 per cent method (9).

† Based on 8 mice per twofold dilution.

§ Based on 5 (Breinl) or 10 (Wilmington) rats per twofold dilution.

|| Based on 8 rats per fourfold dilution.

III). The results of two such experiments, one with a yolk sac pool infected with the Wilmington strain of murine typhus rickettsia and the other with a pool infected with the Breinl strain of epidemic typhus rickettsia are presented in Table IV and are typical of those obtained. With due regard for the limits of error in the various titration methods employed, the parallelism observed between the decline of the several activities measured (time-temperature effect) and their respective increases (as the result of concentration by centrifugation) is striking.

Observations on the Mechanism of the Hemolytic Reaction

It has been deduced that the hemolytic principle is intimately associated with the actual rickettsial bodies. Since these bodies are visible with the aid of a

microscope, it seemed possible that examination of Giemsa-stained smear preparations of hemolyzing yolk sac-substrate mixtures taken after various intervals of incubation would reveal something of the mechanism involved. Except in the 0-hour preparation, in which some indication of the attachment of rickettsiae to the cell margins was seen, the examination of a series of such smears revealed no relation whatsoever between cells and rickettsiae. The principal finding was that in smears taken after progressively longer intervals of incubation the red cells became less abundant and presented evidence of lytic changes with pale, indistinct margins. Quantitative evidence of the progressive reduction in the number of cells present was obtained by several series of counts made by the usual clinical laboratory technique at various intervals after combining yolk sac and substrate. These counts are shown in Table V. In spite of irregu-

TABLE V
Red Cell Counts on Hemolyzing Yolk Sac—Substrate Mixtures after Different Intervals of Incubation

Experiment No.	Rickettsial strain	Dilution of yolk sac	Red cell count (millions) at intervals of incubation (35°C.)						
			0	5-15 min.	30 min.	1 hr.	2 hrs.	4-5 hrs.	18 hrs.
1	Epidemic	1:2	0.73	0.60	0.62	0.62	0.25	0.20	0.15
2	Murine	1:2	2.09	2.01	1.50	—	1.22	0.65	—
		1:4	—	2.33	1.51	—	1.25	0.90	—
		Blank	—	—	—	—	—	1.90	—
3	Murine	1:2	—	—	1.50	—	0.84	0.70	—
		1:4	—	—	1.46	—	1.09	1.06	—
		Blank	1.37	—	—	—	—	1.66	—

larities, it is evident that the rate of the decline in count was related to the concentration of yolk sac employed, but that even with the most concentrated yolk sac preparation (1:2 dilution) a 50 per cent reduction in count did not occur much before the 4th or 5th hour of incubation.

Although direct microscopic examination failed to reveal a clear relation between cells and rickettsiae, it seemed of interest to determine whether cells once exposed to infected yolk sac would continue to undergo hemolysis when removed from the hemolytic system and, conversely, whether such removal of the cells would have a significant effect upon the hemolytic potency of the remaining portion of the system. In these studies, substrate and serial dilutions of yolk sac infected with rickettsiae of a murine typhus strain were allowed to remain in contact for 15 minutes in a water bath adjusted to 37°C. or to 5°C., after which the cell component and supernatant (yolk sac plus serum and saline of the substrate) were separated by brief, light centrifugation. The washed or un-

washed red cells were resuspended in new supernatant fluid composed of appropriate dilutions of normal yolk sac plus rabbit serum and saline to replace the fluid portion of the substrate, while fresh packed cells were added to the original supernatant. Both new series with the indicated control series were then incubated for 18 hours at 35°C. before reading.

TABLE VI
Studies of Possible Adsorption of Hemolytic Activity by Red Cells

Temperature of preliminary incubation °C.	Fraction*	Final hemolytic titer† (1:x)
35	Control mixture	256
	Supernatant	256
	Red cells	64
5	Control mixture	256
	Supernatant	256
	Red cells	64
35	Control mixture	128
	Supernatant	128
	Red cells	32
	Washed once‡	8

* Equal volumes (1.0 ml.) of substrate (25 per cent cells) and yolk sac dilutions mixed; separated after 15 minutes' incubation by centrifugation at 1,000 R.P.M. for 5 minutes; supernatant tested with fresh cells; cells tested with fresh supernatant prepared with normal yolk sac.

† Dilutions showing a trace of hemolysis after 18 hours' incubation at 35°C.

‡ Cells washed with 3.0 ml. (12 volumes) of saline and recentrifuged.

The results of the studies (Table VI) fail to indicate any marked tendency for the hemolytic principle to be adsorbed onto the red cells. In no case was the titer of the supernatant fluid, after a preliminary exposure to red cells, lower than that of the control series, and in all cases the titer observed with exposed red cells after replacement of infected supernatant material with normal material, was demonstrably lower than that of the control. This is in accord with our failure to observe microscopically any adherence of rickettsiae to the red cells and with the evidence that the hemolytic principle is inseparable from the

living rickettsial organisms. Some hemolysis of exposed red cells did occur, however, after their removal from the infected supernatant fluid, and, indeed, even after a single washing in saline. Such residual hemolysis, after washing, was found only in the case of those cells which had been exposed to high concentrations of rickettsiae (sufficient to result in 4+ hemolysis with control and supernatant material). It is probable that the explanation lies in the nature of the hemolytic phenomenon rather than in any true adsorption.

It has been shown previously that the hemolytic process is a slowly progressive one and is apparently dependent upon the rather gradual infliction of damage on the red cells. What the nature of the effect upon the cell may be is not known, although in one experiment, in which somewhat hypotonic saline was employed, the cells did not exhibit increased fragility. Since the hemolytic principle undergoes destruction at temperatures favoring the demonstration of its presence, the degree of hemolysis observed must be the resultant of two opposing factors: (1) the rate of red cell injury and (2) the rate of "hemolysin" destruction. The rate of red cell injury must be determined, in part, by the concentration of active principle (apparently viable rickettsiae), so that, whereas short exposure to high concentrations will result in some damage to the red cells, prolonged exposure to low concentrations, because of the simultaneous inactivation of "hemolysin," may result in little hemolysis. Accordingly, washing of red cells exposed to high concentrations of rickettsiae will not prevent those cells already extensively damaged from subsequently undergoing lysis, while simple separation of cells from the original supernatant fluid by centrifugation will, in addition, leave behind some active material to carry further the damage initiated during the preliminary exposure period. It can be seen that it is impossible to quantitate the results obtained, even approximately, in view of the inadequacy of our knowledge concerning the factors governing the rates of reaction. The fact that some but not all the erythrocytes undergo hemolysis with any preparation is probably due to variations in susceptibility among the cell population and to loss of activity of the preparation occurring before the more resistant cells have been sufficiently affected to undergo lysis.

The Inhibition of Hemolysis with Immune Serum

As has been indicated, early studies of the hemolytic principle showed that its activity could be inhibited by homologous antiserum. In order to investigate this inhibition more fully the optimal conditions for its demonstration had to be determined. A detailed presentation of this preliminary phase of the work will be omitted in favor of data showing the application of the technique to serological studies. In summary, the best results were obtained when equal volumes (0.2 ml.) of infected yolk sac suspensions and dilutions of immune serum, made with saline, were mixed and allowed to remain in contact for 30

minutes at room temperature prior to the addition of the red cell substrate (0.5 ml.). The final mixture was then incubated for 18 hours at 35°C. Simultaneously, control tubes were prepared which contained saline instead of the serum dilutions. The use of too great a concentration of the hemolytic factor resulted in specific serum inhibition titers which were unduly low. Exceedingly weak concentrations, on the other hand, operated to diminish the specificity of the test by permitting the demonstration of inhibitory activity with normal sera. The best compromise was obtained when dilutions of yolk sac giving 2+ or 1 to 2+ hemolysis were employed against serial dilutions of immune serum. It may be recalled at this point that sera of species other than the rabbit occasionally cause hemolysis of rabbit cells and that this generally can be avoided by heating the sera at 56°C. for $\frac{1}{2}$ to 1 hour prior to their incorporation in the test. The very occasional persistence of this activity after heating makes it advisable to use control mixtures of serum and cells to indicate false reactions.

Some suggestion was obtained that the addition of fresh normal guinea pig serum (unheated) somewhat enhanced the antisera titers. The possible rôle of complement in the process was thus indicated, but the frequent hemolytic action of unheated guinea pig serum upon rabbit cells made the addition inadvisable as a routine procedure.

Because of an unexplained difficulty in the production of satisfactory yolk sac pools of strains of murine typhus rickettsiae, most of our experience with the serum inhibition technique has been with the rickettsiae of epidemic typhus. A few satisfactory tests have been made with murine typhus rickettsiae, however, and some of the results obtained with both types are shown in Table VII. The data presented indicate the specificity of the test when applied to cotton rat antisera obtained against either rickettsial strain. Complete inhibition was shown with homologous antisera in dilutions of 1:16 to 1:32 and partial inhibition in dilutions of 1:128 or higher. In contrast, complete inhibition was rarely observed with either normal or heterologous sera, and the degree of partial inhibition was not marked. With rickettsiae of epidemic typhus and sera of humans convalescent from this disease, titers comparable to those seen with animal sera were obtained, and the normal human sera tested showed little non-specific inhibition. High titers were likewise observed with the convalescent human sera against a "hemolysin" of murine typhus origin, however, so that the strain specificity for human studies remains somewhat open to question. Owing to the lack of available potent preparations of murine typhus rickettsiae and of human antiserum against these rickettsiae, this question of strain specificity could not be investigated more fully.

In Table VIII, results of serum inhibition tests with various human and rabbit sera against yolk sac infected with the rickettsiae of epidemic typhus are compared with the results of complement fixation tests, and when available, toxin neutralization tests obtained with the same sera. The use of complete

inhibition of hemolysis as the criterion of activity results in a test of low sensitivity in comparison with other serological methods. However, with the acceptance of partial inhibition as the index, the sensitivity of the technique is

TABLE VII
Antihemolytic Titers of Human and Animal Sera

Rickettsial strain of hemolysin*	Sera†	Antihemolytic titers (1:×)	
		Complete‡	Partial
Murine	Murine (cotton rat)	32	>128
	Epidemic (" ")	Undiluted	16
	Normal (" ")	0	2
Epidemic	Epidemic (cotton rat)	16	128
	Murine (" ")	0	16
	Normal (" ")	0	0
Epidemic	Epidemic (human No. 1)	32	>256
	" (" " 2)	8	16
	" (" " 3)	16	128
	" (" " 4)	32	>256
	" (" " 5)	32	128
	" (" " 6)	8	32
	" (cotton rat)	16	>64
	Normal (" ")	Undiluted	4
	" (human)	0	8
Murine	Epidemic (human No. 2)	64	128
	" (" " 4)	64	128
	" (" " 5)	>256	>256
	" (" " 6)	8	16
	" (cotton rat)	2	32
	Murine (" ")	32	128
	Normal (" ")	0	0

* Hemolysin preparations were dilutions of yolk sac suspensions giving a 2 + hemolysis in the absence of test sera.

† The cotton rat antisera were pools obtained from animals surviving infection with the indicated strain and bled 2 to 3 weeks after inoculation. The human antisera were obtained in 1941 from Spanish prisoners convalescent from typhus fever and were stored under sterile conditions at 4°C. until used.

‡ Highest dilution of serum showing complete inhibition of all hemolytic activity.

|| Highest dilution of serum showing significant reduction in degree of hemolysis as compared with control containing saline instead of serum.

comparable to that of the method of complement fixation employed (10), except in the case of the sera from vaccinated rabbits, in which, particularly as related to adjuvant vaccine (11), a tremendous discrepancy occurred. In the rabbit

TABLE VIII
Comparison of Hemolysin Inhibition, Complement Fixation, and Toxin Neutralization by Immune Sera (Epidemic Typhus Antigens Only)

Serum				End-point dilution (1:x) of serum as measured by			
Species	Individual	History	Time interval	Inhibition of hemolysis*		Complement fixation†	Toxin‡ neutralization
				Complete*	Partial*		
Human	D. C.	Post-typhus vaccination	2 wks.	1	64	50	—
		Laboratory infection (murine)	21 days	1	32	128	—
	J. F.	Laboratory infection (vaccinated)	21 days	1	64	128	—
			31 "	1	64	128	—
	J. S.	Post-typhus vaccination	2 wks.	1	32	24	—
		Laboratory infection (epidemic)	7 mos.	8	128	128	—
O. P.	Laboratory infection type unknown (vaccinated)	15 days	8	128	512	—	
		40 "	16	256	512	—	
C. A.	Post-typhus vaccination	2 wks.	4	64	16	—	
	Laboratory infection (epidemic)	14 days	32	> 512	1500	—	
		5 mos.	8	128	190	—	
Rabbit	No. 21	Adjuvant vaccine (epidemic)	8 wks.	2	> 128	1240	570
			13 "	4	> 128	840	900
			20 "	8	64	400	250
			35 "	1	32	190	250
	No. 37	Saline vaccine (epidemic)	3 wks.	1	2	24	3
			8 "	0	1	21	0
			13 "	0	0	15	0
			35 "	0	0	15	0

* See preceding table.

† 50 per cent hemolysis end-point (10) with 2 units of antigen and 2 full units of complement.

‡ Calculated by the 50 per cent end-point method (9); measured against ± 2.5 LD₅₀ of toxic yolk sac.

|| Vaccine incorporated in water-in-oil emulsion (11).

series, also, the toxin neutralization titers far surpassed the inhibition titers based on either criterion. The number of tests performed up to the present

time has not been sufficient to indicate the true significance of the phenomenon of inhibition.

*Note on the Mechanism of the Toxicity of Rickettsia-Infected
Yolk Sac Suspensions for Mice*

The possible relation of the phenomenon of hemolysis to that of the toxicity of rickettsia-infected yolk sac was suggested naturally by the high degree of parallelism observed in the respective properties of the "hemolysin" and the "toxin" and also by the fact that sera from mice bled during the acute phase of infection with murine typhus rickettsiae (in a study of the development of immunity) consistently evidenced a high content of dissolved hemoglobin. In spite of the fact that mouse red blood cells, *in vitro*, appeared to resist hemolysis, it seemed possible that different conditions *in vivo* might permit hemolysis to occur.

Mice were inoculated intravenously with two or three dilutions of yolk sac richly infected with rickettsiae, with the intention of bleeding the animals at intervals and following, by serial determinations, their blood hematocrit readings and the hemoglobin content of their plasma. The results were unexpected in that the principal toxic effect appeared to be a marked reduction in blood volume. To begin with, it proved nearly impossible to obtain more than 0.1 to 0.2 ml. of blood from the heart of a mouse with signs of toxicity, whereas volumes of from 0.5 to 1.0 ml. were ordinarily taken from normal mice. This difficulty was shortly explained when the cell volumes had been obtained on pooled specimens of heparinized blood. Whereas the cell volumes from normal mice were from 4.0 to 4.5, readings of 5.5 to 7.2 were obtained in several experiments with blood drawn from mice near death following toxic inocula. That the mechanism of toxicity is due to a seriously altered vascular permeability is indicated by the fact that, in spite of the increased cell concentration, the specific gravity of the plasma remained essentially unchanged, ranging from 1.018 to 1.022 in normal mice and in those with toxic signs alike, as determined by the copper sulfate method (12).

Death of the mice following toxic inocula thus was apparently a shock phenomenon. Subsequent experiments were made with animals given additional fluid (2.0 ml. of saline intraperitoneally) or such antihistamine drugs as adrenalin or pyribenzamine. None of these measures affected either the survival of the mice or the hematocrit readings obtained. However, mice inoculated with rickettsia-infected yolk sac mixed with homologous antiserum not only survived but showed no blood changes.

From these experiments it appears that toxicity of rickettsia-infected yolk sac for mice is dependent upon a factor which gravely alters vascular permeability. Whether or not it is this same factor which causes *in vitro* hemolysis

of the blood of other animal species by altering the permeability of the membranes of red cells, is still undetermined.

DISCUSSION AND SUMMARY

Suspensions of yolk sac infected with rickettsiae of murine or of epidemic typhus, and indeed suspensions of liver or peritoneal washings from cotton rats infected with these organisms, when the suspensions contain a sufficient concentration of living rickettsiae, possess the capacity to hemolyze, *in vitro*, red cells of rabbit or sheep origin. Under the same conditions such material did not cause the hemolysis of cells from mice, cotton rats, or guinea pigs. Yolk sac and tissue suspensions of *R. orientalis* (Karp, Raub, and Gilliam strains) failed to hemolyze rabbit cells.

The hemolytic principle was destroyed by formol (0.5 per cent) and by heating to 56°C. for 1 hour. The close association of the "hemolysin" with the rickettsiae was indicated by their parallel movements on centrifugation and by the close parallel demonstrated between the effects produced on hemolytic activity and on such manifestations of the presence of living rickettsiae as toxicity for mice and lethality or total infectivity for cotton rats when infected yolk sac suspensions were exposed to elevated temperature or were concentrated by high-speed centrifugation.

The mechanism of hemolysis remains ill defined. It progresses slowly, but never to completion (*i.e.* hemolysis of all the cells in the substrate) even when the number of such cells is small. This may be because hemolysis progresses best at temperatures ($\pm 35^{\circ}\text{C}.$) which are unfavorable to the hemolytic principle and because erythrocytes vary in their susceptibility to the injurious agent. The concentration of red cells also may be a determining factor, either by affecting the rate at which the reaction proceeds or by aiding in the preservation of hemolytic activity. Although it was impossible to separate the "hemolysin" from the rickettsiae, examination of smear preparations of hemolyzing mixtures showed no consistent contact relation between cells and rickettsiae. Adsorption experiments revealed that, while removal of the cells from the hemolytic system did not reduce the hemolyzing capacity of the system, the cells carried away a significant tendency to hemolyze. It is suggested that the rate at which injury is inflicted on the red cells is dependent on the concentration of "hemolysin" present and that the process may proceed in the relative absence of rickettsiae once sufficient damage has been inflicted.

The toxicity of richly infected yolk sac suspensions for intravenously inoculated mice was found to be related, not to *in vivo* hemolysis, but apparently to a serious alteration in vascular permeability which caused a marked reduction in blood volume. This was evidenced by an increased concentration of red cells but not of plasma proteins. It is possible, but has not been demonstrated,

that the factor responsible for altering vascular permeability of mice may be identical with that which causes lysis of rabbit and sheep cells.

Two applications of the phenomenon of *in vitro* hemolysis are indicated. Following the technique described in the text for the optimum demonstration of hemolysis, the determination of the degree of hemolytic activity may serve as a rapid, roughly quantitative *in vitro* method for assessing the infectivity of a newly made rickettsial preparation. The phenomenon also is the basis for an additional, and potentially useful *in vitro* serologic technique which differs from other techniques in that it depends on a property of living rickettsiae. It has been shown that homologous antisera are capable of inhibiting hemolysis, and optimum conditions for demonstrating this inhibitory effect are described.

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