ROLE OF ADENOSINE TRIPHOSPHATE IN THE HEMOLYSIS OF SHEEP ERYTHROCYTES BY TYPHUS RICKETTSIAE[1]

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Hemolysis by typhus rickettsiae was first reported by Clarke and Fox (1948), who found that homogenates of yolk sacs infected with these organisms brought about a slow lysis of sheep and rabbit erythrocytes at 37 C. Snyder et al. (1954) later described a faster spectrophotometric method for measurement of hemolysis by typhus rickettsiae, and showed that the hemolytic activity of different preparations of typhus rickettsiae, both crude and purified, paralleled their toxicity for mice. They also found that hemolysis by typhus rickettsiae required the presence of glutamate and Mg and was prevented by such metabolic inhibitors as cyanide and fluoride.

More recent work has shown that hemolysis carried out in the presence of glutamate, as originally described, is inhibited by DNP,[2] and that frequently ATP can serve a partial substitute for glutamate. The amount of hemolysis is lowered when glutamate is replaced by ATP, but such hemolysis as does occur is no longer inhibited by cyanide or DNP. It was also noted that rickettsiae subjected to certain procedures that lead to a reversible loss of respiratory activity (as well as to loss of toxicity and infectivity) could no longer hemolyze in the presence of glutamate, but could do so in the presence of ATP. These results suggested that ATP may be involved in some essential capacity in the hemolytic reaction; fully viable rickettsiae being able to form their own ATP in the presence of glutamate, those that had lost the ability to oxidize glutamate needing an external source of ATP. Additional variations of this phenomenon, that cannot be explained solely in terms of this hypothesis, have been found in the response of different types of rickettsial preparations to added ATP.

The hemolytic reaction has therefore been studied with respect to the ability of various preparations of rickettsiae to produce hemolysis in the presence of the following substrates: (1) glutamate, which can serve as a source of internally generated ATP; (2) externally added ATP, usually with KCN added also to prevent generation of ATP; and (3) glutamate and ATP together. With regard to the relative amount of hemolysis produced under these three conditions, the rickettsiae fell into four categories: (A) undamaged, or minimally damaged and later restored rickettsiae, which were characterized by highly active hemolysis with glutamate, very low hemolysis with ATP, and strong inhibition of glutamate hemolysis by added ATP; (B) partially damaged rickettsiae, characterized by less hemolysis with glutamate alone than the undamaged rickettsiae, fairly good hemolysis with ATP alone, and mild inhibition of glutamate hemolysis by ATP; (C) more seriously damaged rickettsiae, characterized by loss of all ability to generate ATP, and showing complete loss of ability to hemolyze with glutamate, and fair ability to hemolyze with added ATP; and (D) starved rickettsiae, characterized by loss of all ability to hemolyze with either added ATP, glutamate, or both, the loss in activity being reversible under suitable conditions.

MATERIALS AND METHODS

Chemicals and solutions. DPN, CoA, and ATP were obtained from the Pabst Laboratories, GSH from Schwarz Laboratories, and bovine serum albumin (plasma fraction V) from the Armour Laboratories.

K-7 contained 0.122 M KCl, 8.06 mM Na_2HPO_4, 4.0 mM KH_2PO_4, and 7.3 mM NaCl. To prepare

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[2] The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; DPN, diphosphopyridine nucleotide; CoA, coenzyme A; GSH, glutathione, reduced form; DNP, dinitrophenol.

K-7alb, 1 volume 6 per cent serum albumin, adjusted to pH 7, was added to 19 volumes K-7.

K-7.5M contained 0.12 M KCl, 6.2 mM K_2HPO_4, 1.1 mM KH_2PO_4, 0.84 mM $MgCl_2$, 0.045 mM $MnCl_2$, and 0.022 mM $CaCl_2$.

Na-7 contained 0.129 M NaCl, 8.2 mM Na_2HPO_4, and 3.8 mM KH_2PO_4.

Na-7M was the same as Na-7 except for addition of 7.7 mM $MgCl_2$, 0.21 mM $CaCl_2$, and 0.086 mM $MnCl_2$.

Measurement of hemolytic activity. Sheep red cells, from sheep blood collected in one volume 2 per cent citrate weekly, were washed three times with 0.85 per cent NaCl, then suspended in Na-7M to give a 33 per cent suspension of cells by volume.

The rickettsiae were diluted in K-7alb at room temperature immediately before use. All other substances were also diluted in K-7alb to give the desired final concentration. In the usual assay 0.3 ml of sheep cells, 0.2 ml of a suitable dilution of rickettsiae, and 0.1 ml of a solution containing all other desired additions were mixed in an ice bath, the rickettsiae being added last. All were incubated at 34 C for 2 hr, then 2 ml 0.85 per cent NaCl, containing 0.2 per cent formalin, were added to each tube, and the contents were mixed and centrifuged for 10 min at 2000 rpm. The amount of hemolysis was estimated by measurement of optical density at 545 mμ in a Coleman Junior spectrophotometer, each tube being read against a blank which contained K-7alb in place of the rickettsiae. The amount of hemolysis is proportional to the concentration of rickettsiae when other constituents are held constant (Snyder et al., 1954). Several dilutions of rickettsiae were always included so that the amount of rickettsiae required to give an optical density reading of 0.3, arbitrarily chosen as the hemolytic end point, could be estimated graphically. This amount of rickettsiae is termed the hemolytic unit and all results are expressed as hemolytic units per mg rickettsial protein. The protein content of the rickettsiae was determined with the Folin reagent according to Lowry et al. (1951). In most experiments hemolysis was measured under three conditions, as follows: (1) with 0.1 ml of 20 mM glutamate per tube; (2) with 0.1 ml of 6 mM ATP, 6 mM KCN per tube; and (3) with 0.1 ml of 20 mM glutamate, 6 mM ATP per tube. It should be noted that significant variations sometimes occur in the absolute value of the hemolytic activity measured with different lots of sheep cells. Relative values however are not so affected.

Rickettsiae. The E strain of epidemic typhus, grown in the yolk sacs of embryonated eggs, was purified as described by Bovarnick (1956). Toxicity of the rickettsiae was measured as described by Allen, Bovarnick, and Snyder (1954).

Types of rickettsial preparations.

A. *Undamaged rickettsiae.*

1. These were rickettsiae purified from freshly harvested yolk sacs and used at once.

2. Slightly damaged rickettsiae, category B1 below, were restored to the undamaged state by dilution into a medium consisting of K-7.5M, to which had been added 4 mM glutamate, 0.14 mg/ml DPN, 0.5 mM GSH, 0.025 mg/ml CoA, and the amino acid mixture and soluble yolk sac protein fraction described elsewhere (Bovarnick, Schneider, and Walter, 1959), and then incubated at 30 C for 16 to 24 hr. Under these circumstances the infectivity and hemolytic activity with glutamate are well maintained for at least 24 hr.

B. *Partially damaged rickettsiae.* These were produced by two procedures, the first leading to only very slight, the second to somewhat more marked inactivation.

1. Rickettsiae were purified from yolk sacs that had been stored for a time at −70 C, then resuspended in isotonic sucrose at a concentration of 8 to 10 mg protein per ml and usually stored again at −70 C before use. These are probably slightly damaged preparations, although their infectivity, hemolytic activity with glutamate, toxicity, and rate of respiration are usually nearly as high as those of the undamaged rickettsiae. However, their reactivity with ATP differs a little from that of the undamaged rickettsiae, as will be described later, and after incubation in the complete medium their behavior toward ATP changes to that characteristic of the undamaged rickettsiae, and some preparations also show increased hemolysis with glutamate.

2. More obviously damaged rickettsiae were obtained by diluting those obtained by procedure B1 20- to 60-fold into K-7.5M containing 4 mM glutamate, 0.27 mg/ml DPN, and 3 mg/ml soluble protein from yolk sac (Bovarnick et al., 1959), and incubating for 24 to 48 hr at 30 C before measurement of hemolytic activity. Similar preparations were obtained more rapidly by di-

TABLE 1

Lysis of sheep red cells by typhus rickettsiae in the presence of glutamate or ATP

Additions	Substrate	
	Glutamate	ATP
	*units**	*units**
Both glutamate and ATP omitted	18	18
No addition	769	122
DNP, 0.3 mM	131	128
KCN, 1 mM	12	124
KF, 1 mM	29	12
DPN, 2.7 μg/ml	762	131
DPN, 2.7 μg/ml; CoA, 1 μg/ml; GSH, 0.1 mM	810	122

Glutamate and ATP, when present, were used in a final concentration of 3.3 and 1 mM, respectively. The other concentrations given are also those used in the final hemolytic reaction mixture.

* Hemolytic units per mg of protein.

luting the initial rickettsiae only 7- to 8-fold into K-7.5M containing glutamate, but no DPN, and incubating for 4 hr at 34 C with shaking. Such rickettsiae have definitely lower toxicity and hemolytic activity with glutamate than do undamaged rickettsiae and most of the type B1 preparations.

C. *Rickettsiae incapable of oxidative phosphorylation.* These were prepared in two ways.

1. Rickettsiae, prepared as in procedure B1, were diluted 20-fold into Na-7 containing 4 mM glutamate at 0 C and left at that temperature for 24 hr. This treatment leads to loss of all respiration, toxicity, hemolytic activity with glutamate, and much of their infectivity. All of these properties can be restored by subsequent incubation with glutamate, DPN, GSH, and CoA (Bovarnick and Allen, 1957*a*).

2. Rickettsiae were purified from either a fresh or frozen pool of yolk sacs and resuspended in K-7, then frozen and thawed twice. Such rickettsiae have the same characteristics as those treated by procedure C1 (Bovarnick and Allen, 1954).

D. *Starved rickettsiae.* Rickettsiae, prepared as under procedure B1, were diluted 20-fold in K-7.5M containing 0.14 mg/ml DPN and 3 mg/ml serum albumin, and were left for 2¾ hr at 36 C. These rickettsiae have lost all hemolytic activity with glutamate, toxicity, and much of their infectivity. Toxicity and hemolytic activity with glutamate can be restored by subsequent incubation with glutamate at 30 C for 2 to 3 hr (Bovarnick and Allen, 1957*b*).

RESULTS

The general characteristics of the hemolytic reaction in the presence of the two substrates, glutamate and ATP, are shown in table 1, which shows the influence of various coenzymes and inhibitors on hemolysis by washed rickettsiae that had been stored at −70 C in sucrose before use. As already mentioned, in the presence of salts and rickettsiae alone, there is little significant hemolysis. Addition of glutamate allows good hemolysis, and ATP can also support hemolysis to the extent of 16 per cent of that found with glutamate. In other experiments not shown there was slight hemolysis with pyruvate, even less than with ATP, none with succinate, α-ketoglutarate, ADP, UTP, CTP, or GTP. Addition of DPN, CoA, and GSH directly to the hemolysin mixture did not affect the extent of hemolysis with either glutamate or ATP, in contrast to the marked effects described below when rickettsiae are preincubated with these substances. Fluoride strongly inhibited hemolysis with both substrates, but the effect of KCN and DNP was quite different in the two systems, glutamate hemolysis being strongly inhibited, ATP hemolysis entirely unaffected. This last observation made it possible, by inclusion of KCN as well as ATP in the hemolysis reaction mixture, to measure ATP hemolysis even when glutamate could not be excluded completely from the reaction mixtures, as, for instance, when rickettsiae were preincubated with glutamate in some of the procedures outlined in the experimental section.

The hemolysis brought about by variously treated rickettsiae in the presence of glutamate, ATP, and KCN, or ATP and glutamate is shown in tables 2 and 3. Several types of reactivity can be distinguished. At one extreme are the Type A rickettsiae that hemolyze best with glutamate and very poorly with ATP. Their activities with ATP-KCN and with ATP-glutamate are only 5 and 10 per cent, respectively, of their hemolytic activity with glutamate alone. Such behavior is shown by rickettsiae never frozen or stored (table 2, experiment no. 1a), which represent the best approximation to completely undamaged rick-

TABLE 2

Hemolytic activity of typhus rickettsiae after freezing and maintenance at 0 C

Expt No.	Treatment of Rickettsiae	Hemolytic Activity with			Toxicity
		Glutamate	ATP and KCN	Glutamate and ATP	
		*units**			LD_{50}/p
1a	Washed, never frozen	808	36	75	
b	Washed, frozen in sucrose	775	118	225	
2a	Washed from frozen pool	741	103	110	
b	a, Frozen in sucrose	783	109	112	
c	a, Frozen in K-7	21	238	244	
3a	Washed from frozen pool	797	133	303	179
b	a, Frozen in K-7	16	372	352	<7
c	b, Incubated with cofactors, 30 C	339	133	326	57
d	a, Diluted in Na-7 + glutamate, 24 hr, 0 C	6	150	197	<7
e	d, Incubated with cofactors, 30 C	629	266	328	179

The concentrations of glutamate, ATP, and KCN, when present during measurement of hemolytic activity were 3.3, 1.0, and 1.0 mM, respectively.

The figures for toxicity represent the number of LD_{50}, or minimal amount necessary to kill 50 per cent of the mice after intravenous inoculation, per mg protein.

In experiment no. 3c, the frozen rickettsiae were diluted 20-fold into the complete medium described in the experimental section, and kept for 3 hr at 30 C, then assayed. In experiment no. 3d, the original suspension of rickettsiae was diluted 20-fold at 0 C with Na-P7 containing 3 mg/ml serum albumin and 3 mM glutamate, and left at this temperature for 24 hr. For reactivation of 3d, DPN, CoA, GSH, $MgCl_2$, and $MnCl_2$, at the same final concentrations as used in experiment no. 3c were added and the mixture then was incubated for 3 hr at 30 C (experiment no. 3e).

* Hemolytic units per mg of protein.

TABLE 3

Effect of ATP on hemolysis by typhus rickettsiae incubated in various media at 30 C

Expt No.	Conditions of Incubation		Hemolytic Activity with			Toxicity
	Medium	Time	Glutamate	ATP and KCN	ATP and glutamate	
		hr	*units**			LD_{50}/p
4a	No incubation	0	572	214	272	
b	Glutamate and DPN	24	392	400	462	
		48	135	196	327	
c	Glutamate, DPN, and GSH	24	508	87	180	
		48	190	50	75	
d	Complete medium	24	695	43	106	
		48	357	40	52	
5a	No incubation	0	801	78	159	179
b	Glutamate and DPN	24	427	224	502	57
		48	32	136	287	
c	Glutamate, DPN, and GSH	24	667	50	89	
		48	293	12	54	
d	Complete medium	24	695	30	39	179
		48	407	10	41	

The figures for hemolytic activity and toxicity have the same significance as given in the footnote to table 2 and the concentrations of glutamate, ATP, and KCN were also the same.

The rickettsiae used as starting material had been purified from frozen yolk sacs and stored at −70 C as a concentrated suspension in sucrose before use (procedure B1). For experiment nos. 4b and 5b, the rickettsiae were diluted to a concentration of 0.14 mg protein per ml in K-7.5M, to which had been added 4 mM glutamate, 0.14 mg/ml DPN, 0.3 mg/ml soluble protein from yolk sac, and kept at 30 C for the indicated time. In experiment nos. 4c and 5c the incubation medium contained also 0.5 mM GSH. For experiment nos. 4d and 5d, the complete medium described in the experimental section was used (procedure A2).

* Hemolytic units per mg of protein.

ettsiae available, since the high adenosine triphosphatase activity of crude yolk sac homogenates makes it impossible to test such material before purification. Similar behavior (i.e., high activity with glutamate alone, very little with ATP) was shown by type A2 rickettsiae, which had been purified from frozen pools and subse-

quently incubated at 30 C in the complete medium described in the experimental section, before measurement of hemolytic activity (table 3, experiment nos. 4d and 5e).

At another extreme are the rickettsiae showing no activity with glutamate and whose hemolysis with ATP-KCN and with ATP-glutamate is the same, about one-quarter to one-half that found with glutamate in undamaged rickettsiae. Such behavior is shown by the type C rickettsiae, i.e., those frozen and thawed while suspended in isotonic salt solution after purification, or left for 24 hr at 0 C in a medium high in Na and containing glutamate (table 2, experiment nos. 2c, 3b, and 3d). Rickettsiae treated in either of these ways have lost respiratory activity as well as ability to hemolyze with glutamate, the loss in activity being apparently due to loss of DPN.

Intermediate responses to ATP are also found. Thus the usual initial type B1 rickettsial preparation, one that has been frozen first as a 50 per cent yolk sac homogenate, then frozen again in sucrose after purification, has frequently nearly the same hemolytic activity with glutamate as a preparation never frozen or stored. With ATP-KCN hemolysis is 15 to 20 per cent, with ATP-glutamate 20 to 30 per cent of that with glutamate alone (table 2, experiment nos. 1b, 2b, 3b; table 3, experiment no. 5a). Since these preparations have high infectivity, toxicity, etc., the chief reason for considering them to be slightly damaged is that they exhibit appreciably more reactivity with ATP than do rickettsiae never frozen or stored, a change in the same direction as is exhibited by rickettsiae frozen under less favorable conditions. Also occasional preparations made in this way show definitely decreased hemolysis with glutamate, usually accompanied by still higher reactivity with ATP (table 3, experiment no. 4a) and these last preparations, after incubation in the complete medium, show increased ability to hemolyze with glutamate, as well as decreased reactivity with ATP-KCN and ATP-glutamate, as they are restored to the undamaged state.

More regularly reproducible and extensive damage is shown by rickettsiae incubated at 30 C with glutamate and DPN for 24 to 48 hr (type B2). Under such conditions ability to hemolyze

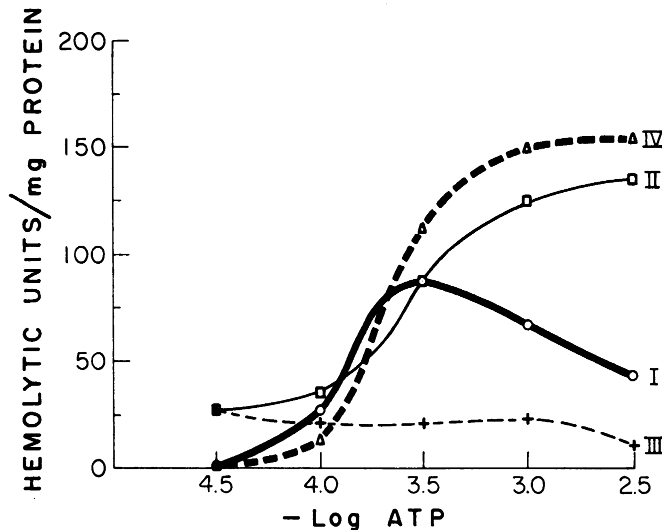

Figure 1. The effect of ATP on hemolysis by various types of typhus rickettsiae in the absence of glutamate. *Curve I*, rickettsiae that had been purified from yolk sacs stored at −70 C and then suspended in isotonic sucrose and again stored at −70 C before use. *Curve II*, rickettsiae purified and stored as *curve I*, then diluted into a medium containing 103 mM KCl, 4.8 mM KH_2PO_4, 10.2 mM K_2HPO_4, 3.2 mM $MgCl_2$, 0.036 mM $MnCl_2$, 0.086 mM $CaCl_2$, 1.6 mg/ml bovine serum albumin, 8 mM glutamate, and 30 mM sucrose (sucrose introduced with the rickettsiae). They were incubated with shaking in this medium for 4 hr at 34 C before measurement of hemolytic activity. *Curve III*, rickettsiae treated as in *curve II*, except that the incubation medium included also 0.27 mg/ml DPN, 0.05 mg/ml CoA, and 1 mM GSH. *Curve IV*, rickettsiae that had been suspended in K-7 after purification and twice frozen and thawed. Hemolysis was measured in the presence of 1 mM KCN and varying concentrations of ATP.

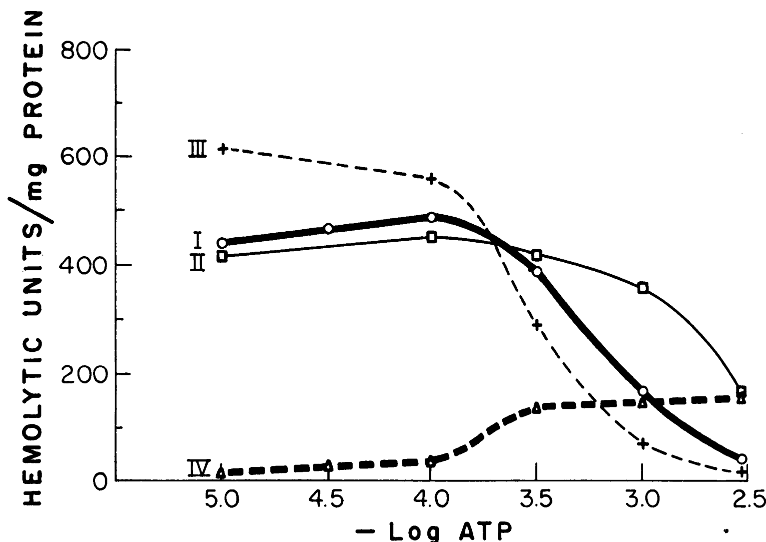

Figure 2. Effect of ATP on hemolysis by various types of preparations of typhus rickettsiae in the presence of glutamate. *Curves I, II, III,* and *IV* represent rickettsiae treated as described in figure 1. Hemolysis measured in the presence of 3.3 mM glutamate and varying concentrations of ATP.

with glutamate alone decreases continuously with time of incubation. Ability to hemolyze with ATP-KCN and ATP-glutamate increases for 24 hr, then decreases. The decline in activity with ATP is slower than the decline in reactivity with glutamate, so that after 48 hr, hemolysis with ATP is greater than hemolysis with glutamate (table 3, experiment nos. 4b and 5b). Rickettsiae so treated also gradually lose toxicity, infectivity, and respiration.

The hemolytic activity of one preparation of each of the extreme types and of two preparations showing differing degrees of partial damage was studied as a function of the concentration of added ATP, both in the absence and in the presence of glutamate, and the results are shown in figures 1 and 2. As an example of undamaged rickettsiae, a type A2 preparation that had been incubated for 4 hr at 34 C with glutamate, DPN, CoA, and GSH was used (curves III). In the absence of glutamate, these rickettsiae show poor hemolysis at all concentrations of ATP. With glutamate, ATP brings about 50 per cent inhibition of hemolysis at a concentration as low as 0.3 mM, and the inhibition increases at higher concentrations.

A slightly damaged preparation of type B1, that is rickettsiae prepared from a frozen pool and stored in sucrose at −70 C, shows with ATP alone, hemolysis that increases to a maximum at 0.3 mM ATP, then declines with further increase in the concentration of ATP. In the presence of glutamate, 0.03 to 0.1 mM ATP produces a slight increase in hemolysis, but higher concentrations lead to a decrease in activity, reaching 50 per cent of the value without ATP at about 0.8 mM (curves I). Rickettsiae further damaged by incubation at 34 C with glutamate, but no cofactors for 4 hr, type B2, show with ATP alone hemolysis that increases with the concentration of ATP up to the highest concentration tested, 3 mM. In the presence of glutamate there is little inhibition by ATP until the concentration reaches 2 to 3 mM (curves II).

Rickettsiae completely inactivated with respect to glutamate hemolysis, but still capable of hemolysis with ATP, type C, show the same degree of hemolysis with or without glutamate, and hemolysis increases with increase in the concentration of ATP up to 3 mM ATP (curves IV).

In the instances so far considered poor hemolysis with ATP-KCN has been associated with sensitivity of hemolysis to inhibition by ATP, and a high degree of such sensitivity, combined with good hemolysis in the presence of glutamate alone, seems to be characteristic of the undamaged rickettsiae. Inactivation of the rickettsiae with respect to glutamate hemolysis (and respiration, toxicity, and infectivity) has been associated with increasing hemolysis in the presence

of ATP-KCN and decreasing sensitivity of the remaining glutamate hemolysis to inhibition by ATP. This suggests that the paradoxical increase in hemolytic activity with ATP shown by the damaged rickettsiae is a consequence of their decreased sensitivity to inhibition by ATP. The inhibition of hemolysis of undamaged rickettsiae by ATP was quite specific, in that UTP, CTP, and GTP were without effect and ADP only slightly inhibitory, due possibly to some formation of ATP from the latter in the test system.

TABLE 4

Hemolytic activity of typhus rickettsiae after starvation at 36 C

Expt No.	Conditions of Incubation			Hemolytic Activity with		
	Medium	Temp	Time	Glutamate	ATP and KCN	ATP and glutamate
		C	*min*	*units**		
6a	No incubation		0	490	97	167
b	DPN, no glutamate	36	165	5	<4	21
c	b, Glutamate added after 165 min	30	180	365	65	191
d	Complete medium, except no glutamate	36	165	16	4	5
e	d, Glutamate added after 165 min	30	180	382	9	23

The figures for hemolytic activity have the same significance as given in the footnote to table 2 and the concentrations of glutamate, ATP, and KCN used in measurements of hemolysis were also the same.

The rickettsiae used as starting material had been prepared from frozen yolk sacs and stored at −70 C after purification as a concentrated suspension in isotonic sucrose before use. For 6b they were diluted, giving a final concentration of 0.42 mg protein per ml, into a solvent similar to K-7.5M, except for replacement of the phosphate buffer by glycylglycine, pH 7.5. Serum albumin, 3 mg/ml, and DPN, 0.14 mg/ml, were also added. The mixture was incubated for 165 min at 36 C before assay. In experiment no. 6d, the rickettsiae were similarly treated, except that the medium included GSH, CoA, and all of the naturally occurring amino acids except glutamate, at the same concentrations as used in the complete medium. At the end of the starvation period, 7 mM glutamate, 6 mM K phosphate, pH 7.5, and 0.4 mM adenylic acid were added to each, giving 6c and 6e, respectively, and these were incubated for another 3 hr at 30 C.

* Hemolytic units per mg of protein.

Despite this inhibitory property of ATP, it seems safe to conclude that ATP is always necessary for hemolysis and that it is a more immediately essential substrate than is glutamate. This is suggested by the effect of the metabolic inhibitors on hemolysis, as well as by the known characteristics of the different types of rickettsiae used. Thus glutamate hemolysis is completely inhibited by cyanide and DNP, whereas ATP hemolysis is unaffected by these compounds. The undamaged rickettsiae, and those only slightly damaged by freezing in sucrose, are known to contain and be able to form ATP in the presence of glutamate at 30 to 34 C, and to be incapable of forming ATP in the presence of cyanide or DNP (Bovarnick, 1956; Bovarnick and Allen, 1957*b*). On the other hand, rickettsiae that have been frozen in a salt solution, or kept for 24 hr at 0 C in a solution high in Na and containing glutamate, i.e., rickettsiae that can no longer hemolyze with glutamate but can do so with ATP, have lost all ability to respire due to loss of DPN, and are therefore incapable of forming their own ATP. Thus it appears that internally generated ATP, available to undamaged and to partially damaged rickettsiae through oxidative phosphorylation, is the most effective substrate for hemolysis, but must be replaced by added ATP for rickettsiae no longer capable of oxygen uptake and phosphorylation.

There are, however, a few instances in which rickettsiae that have been partially or completely inactivated by relatively mild methods show no increased reactivity with ATP. If rickettsiae are left for 3 hr at 36 C in the absence of substrate (type D), hemolytic activity with ATP is lost as completely as is hemolytic activity with glutamate, and there is no reason to believe that in this instance ATP was inhibitory, since the only slight activity observed was in the presence of both glutamate and ATP. If the starvation is carried out in a medium containing only DPN and salts, subsequent incubation with glutamate leads to recovery of ability to hemolyze with both glutamate and ATP (table 4, experiment nos. 6b and 6c). If a more nearly complete medium, including CoA and GSH as well as DPN, is used

during starvation, both types of hemolysis are again lost, but only glutamate hemolysis can be recovered, since the reactivated rickettsiae are highly sensitive to inhibition by ATP (table 4, experiment nos. 6d and 6e). That ability to hemolyze with ATP should be as completely lost on starvation as ability to hemolyze with glutamate is in accord with the previous finding that within a few minutes after addition of glutamate to starved rickettsiae, their ATP content rises from zero to its maximal value, although in this interval little of the lost hemolytic activity has been restored (Bovarnick and Allen, 1957*b*). Quite obviously in this instance therefore, in contrast to types of inactivation due primarily to loss of DPN, lack of ability to form ATP could not be the cause of the loss of activity, and added ATP could not be expected to restore activity. The nature of the reversible change that takes place on starvation and leads to loss of all hemolytic activity, also to loss of toxicity, is not known.

Finally there is one other case in which loss of activity with glutamate is associated with no increase in activity with ATP. If rickettsiae are incubated in the complete medium described above at 30 C for 24 hr, they remain, as already mentioned, completely undamaged, and the slight damage produced by freezing in sucrose is even repaired. However on longer incubation inactivation is observed, as evidenced by a decline in hemolysis with glutamate, and this decline is not accompanied by any decrease in sensitivity to inhibition by ATP. Reactivity with ATP-KCN declines therefore as rapidly as does reactivity

TABLE 5

Effect of ATP on the rate of oxygen uptake by typhus rickettsiae

Expt No.	Pretreatment of Rickettsiae	Additions during Measurement of Oxygen Uptake	Rate of Oxygen Uptake			Hemolytic Activity		
			−ATP	+ATP	Ratio, +/− ATP	−ATP	+ATP	Ratio, +/− ATP
			µl/hr/p			units*		
6	20 hr, 30 C, with DPN and glutamate	Glutamate	23	19	0.84	340	404	1.2
		Glutamate, DPN, CoA, and GSH	26	23	0.88			
	20 hr, 30 C, in complete medium	Glutamate	30	21	0.68	533	38	0.07
		Glutamate, DPN, CoA and GSH	35	21	0.58			
7	2½ hr, 34 C, with glutamate	Same	31	24	0.77	402	307	0.76
	2½ hr, 34 C, with glutamate, DPN, CoA, and GSH	Same	44	27	0.62	628	50	0.08

In experiment 6, the rickettsiae were diluted and left at 30 C in the media described for experiments 4b and 4d, table 3. After 20 hr at 30 C, the mixtures were assayed for hemolytic activity, then centrifuged for 10 min at 10,000 rpm. The rickettsiae were resuspended in 0.22 M sucrose-0.01 M phosphate, pH 7.5 to a concentration of 6.5 mg protein per ml and these suspensions were used for measurement of the oxygen uptake.

In experiment 7, the rickettsiae were added directly to the Warburg flasks. After 2½ hr at 34 C, ATP was added to half of the flasks and shaking was continued for 1 hr longer, when those lacking ATP were assayed for hemolytic activity.

Measurements of oxygen uptake were made at 34 C, each flask containing the following: 0.2 ml rickettsial suspension in isotonic sucrose, 0.2 ml 0.1 M K phosphate, pH 7.5, 0.04 ml 6 per cent serum albumin, 0.05 ml of a mixture of 0.096 M $MgCl_2$, 0.0025 M $CaCl_2$, and 0.00107 M $MnCl_2$, and 0.075 ml 0.16 M glutamate. Where indicated, 0.075 ml DPN, 5.4 mg/ml, and 0.075 ml CoA, 1 mg/ml, GSH, 0.02 M, were also added. The final volume was brought to 1.5 ml with 0.15 M KCl.

Rate of oxygen uptake is given as the µl per hour per mg rickettsial protein. Hemolytic activity is given as in the other tables. The dilution necessary for measurement of hemolytic activity was sufficient so that none of the substances present during incubation had any effect on the hemolytic reaction itself.

* Hemolytic units per mg of protein.

with glutamate (table 3, experiment nos. 4d and 5d). GSH appears to be the most important single factor in preserving sensitivity to inhibition by ATP, since rickettsiae kept with only GSH, DPN, and glutamate are almost as sensitive as those kept in the complete medium, in marked contrast to those kept with DPN and glutamate (table 3, experiment nos. 4c and 5c). There is reason to believe that this inactivation that very slowly occurs above 30 C in the complete medium differs in one fundamental respect from all of the other kinds of inactivation described here, as will be discussed later.

It should be mentioned that hemolysis with glutamate alone shows a close correlation with the toxicity of the different preparations for mice, whereas no such correlation exists between toxicity and hemolysis in the presence of ATP. This was apparent from numerous examples in earlier work of similarity between changes in toxicity and glutamate hemolysis, and is further illustrated by the toxicity of a few of the preparations used here, as shown in the last columns of tables 2 and 3.

It seemed of interest to determine whether the strong inhibition by ATP of hemolysis by undamaged rickettsiae would be paralleled by any effect of ATP on their respiration. Sensitive (type A2) rickettsiae were obtained by preincubation of the rickettsiae at 30 C in a complete medium. For comparison another sample of the same pool was incubated at 30 C in the absence of GSH and CoA to obtain a preparation (type B2) whose hemolysis would not be inhibited by ATP, yet would still have a reasonable respiration. It was found that the respiration of both types was inhibited by ATP to a small extent, the former being the more sensitive (table 5). However, the difference in the sensitivity of the respiraton of the two types was minor compared to the difference in the sensitivity of their hemolytic activity.

DISCUSSION

Any interpretation of the role of ATP in rickettsial hemolysis is obviously complicated by the great variation in response of the hemolytic activity to addition of ATP. A single concentration of ATP may produce anything from a 10-fold increase in hemolysis to a 90 per cent inhibition, depending upon the pretreatment of the rickettsiae and the other substances present during the assay. This variability is due to the fact that ATP is at the same time essential to hemolysis and a strong inhibitor of hemolysis, and to the further complication that the sensitivity to inhibition by ATP varies with the pretreatment of the rickettsiae. The inhibitory effect of ATP does not necessarily contradict the assumption that ATP is more nearly a direct reactant in hemolysis than is glutamate, for inhibition by ATP of reactions in which it is required is fairly common. In many reactions a small amount of ATP together with an ATP generating system is more effective than is a high concentration of ATP. The undamaged rickettsiae, which are highly sensitive to inhibition by ATP, constitute just such an ATP generating system in the presence of glutamate. However, rickettsial hemolysis differs from other ATP requiring systems sensitive to inhibition by excess ATP in that the sensitivity of the rickettsial reaction is variable. Thus as the activity of the rickettsiae, with respect to respiration and ability to hemolyze in the presence of glutamate, is gradually lowered by certain treatments described above, the sensitivity of their remaining hemolytic activity to inhibition by ATP is progressively lowered, the loss in sensitivity being sufficient to make possible an actual increase in the hemolysis observed in the presence of relatively high concentrations of added ATP. It might seem that the conclusion that damage to the rickettsiae results in loss of sensitivity to inhibition by ATP is contradicted by one instance when loss of ability to hemolyze with glutamate is not accompanied by any such loss in sensitivity. When rickettsiae are incubated in the complete medium at 30 C, ATP strongly inhibits hemolysis not only after 24 hr, when hemolysis with glutamate is still high, but also after 48 hr, by which time hemolysis with glutamate has begun to decline. It seems probable that in this case the decline in activity is due to the successive and complete destruction of individual rickettsiae, those that remain being fewer in number, but unchanged in all of their characteristics, i.e., they are still what we have termed "undamaged." On the other hand the other methods of inactivation used, leading to what have been referred to as "damaged" rickettsiae, probably bring about a defined limited change in the majority of the rickettsiae, all being affected similarly and more or less simultaneously. This must certainly be true of rickettsiae that have been inactivated to the extent that all measurable toxicity and he-

molysis with glutamate have been lost, but which can later regain a large portion of the lost activity. Such a limited change in all of the rickettsiae would make possible the qualitative changes in the behavior of the hemolytic reaction that have been observed in so many instances, as opposed to the purely quantitative changes to be expected after total destruction of a small or large fraction of the rickettsiae.

The extreme sensitivity of hemolysis by undamaged rickettsiae to inhibition by ATP is in itself an interesting phenomenon which cannot be related to any other known effect of ATP on the rickettsiae. Their oxygen uptake is slightly lowered by ATP, but the change in respiration is small compared to the 90 per cent inhibition of hemolysis brought about by a similar concentration of ATP. Also their stability is not adversely affected by ATP, and ATP is actually required for amino acid incorporation by such rickettsiae, even in the presence of glutamate (Bovarnick et al., 1959). Thus ATP appears to inhibit only the hemolytic activity of these undamaged rickettsiae, and it is possible that this inhibition actually serves a useful purpose. The observation of Cohn et al. (1959) that penetration of rickettsiae into mouse lymphoblasts, cells in which they eventually grow, is increased by glutamate and depressed by cyanide and DNP, suggests that this process may be controlled by the same factors that control the hemolytic reaction, and may therefore have a similar mechanism. If penetration involves a local increase in permeability of the cell membrane, it might well be advantageous that such a reaction cease as soon as the rickettsiae have entered the host cell to avoid premature lysis of the host. A reaction inhibited by ATP should automatically stop inside any normal cell.

The indication that ATP is involved in some essential capacity in hemolysis by rickettsiae raises the question as to whether there is any relation between this hemolytic reaction and the one studied by Borek and Bovarnick (1956, 1959), in which red cells are hemolyzed in the presence of a soluble protein fraction from rabbit erythrocytes and ATP. The two systems differ in many respects, and in fact the only known resemblance is the requirement for ATP and Mg and the sensitivity to fluoride. Certainly some differences are to be expected between a soluble protein and an organized cell, and since the basic mechanism of neither reaction is understood, it is impossible at the moment to decide whether ATP can participate in two different hemolytic reactions or only one.

SUMMARY

The effect of adenosine triphosphate (ATP) on hemolysis by typhus rickettsiae varies markedly with the nature of the pretreatment of the rickettsiae and with the nature of the other substances present during the assay. With undamaged rickettsiae, that is those never frozen or stored, or those kept at 30 C in a medium containing glutamate, diphosphopyridine nucleotide, coenzyme A, and reduced glutathione, hemolysis with glutamate is very good, but this hemolysis is strongly inhibited by ATP and with ATP alone there is very little hemolysis. Partially damaged rickettsiae, i.e., those stored frozen in sucrose for a time before use, or rickettsiae kept at 30 C without reduced glutathione or coenzyme A, show progressive loss of ability to hemolyze with glutamate, decreasing sensitivity to inhibition by ATP, and increasing hemolysis with ATP alone, though the reactivity with ATP never reaches more than 30 to 40 per cent of the activity found with glutamate alone in undamaged rickettsiae. Rickettsiae that have been completely but reversibly inactivated with respect to most of their properties, including the ability to phosphorylate, as by freezing in an isotonic salt solution or by maintenance at 0 C in a solution high in Na and containing glutamate, can no longer hemolyze with glutamate, but can do so with ATP, again less efficiently than was possible with undamaged rickettsiae in the presence of glutamate.

If rickettsiae are starved at an elevated temperature, ability to hemolyze with either ATP or glutamate is lost, although this may be regained by subsequent incubation with glutamate. In this case it is known that lack of ability to form ATP could not be the cause of the lost activity.

Hemolysis that takes place in the presence of glutamate is completely inhibited by cyanide and dinitrophenol. Hemolysis in the presence of ATP alone is unaffected by these two compounds.

It is concluded that ATP is always essential for rickettsial hemolysis, internal ATP generated during the oxidation of glutamate being used by undamaged rickettsiae, external ATP being necessary for hemolysis by rickettsiae that have lost the ability to generate their own ATP. With undamaged rickettsiae the strong inhibition of

hemolysis by externally added ATP is the probable cause of their poor reactivity with added ATP.

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