REVERSIBLE INACTIVATION OF TYPHUS RICKETTSIAE AT 0 C[1]

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Reversible inactivation of typhus rickettsiae was first demonstrated with organisms that had been inactivated by freezing and thawing in isotonic salt solution. As previously described (Bovarnick and Allen, 1954), such treatment led to extenisve loss of mouse toxicity, hemolytic activity, respiration, and infectivity for eggs, and rendered the DPN[2] of the rickettsiae completely diffusible. The loss of DPN and possibly of CoA appeared to be the cause of at least a portion of the loss in all types of activity, since incubation of the inactivated organisms with DPN and CoA restored a significant proportion—10 to 30 per cent—of the lost activities.

It has since been found that a similar but more completely reversible type of inactivation takes place when the rickettsiae are left overnight at 0 C. The extent of this inactivation is dependent on a variety of factors, but in every case between 50 and 100 per cent of the initial activity can be restored by subsequent incubation at a higher temperature in a balanced salt solution containing glutamate and DPN. Although reactivation cannot be brought about in the absence of DPN, there is evidence that loss of this substance alone from the organisms cannot account for all of the changes in activity that take place at 0 C.

EXPERIMENTAL METHODS

Materials and media. DPN was obtained from the Sigma Chemical Company and was stated to be 90 per cent pure. CoA was obtained from the Pabst Laboratories. The serum albumin was Armour's bovine albumin powder. The isotonic salt solution used in washing the rickettsiae, referred to as K-7, contained 0.118 M KCl, 0.0072 M NaCl, 0.01 M phosphate, pH 7.0. The isotonic sucrose solution used for storage at −70 C and also in some instances for washing the rickettsiae, referred to as sucrose P, contained 0.22 M sucrose and 0.01 M potassium phosphate, pH 7.1.

The media used during the 0 C inactivation experiments contained NaCl and KCl in varying proportions, the total chloride concentration being 0.137 M, 0.008 M phosphate, pH 7.0, unless otherwise indicated, and 0.3 per cent bovine serum albumin. Where indicated, 0.005 M glutamate, 0.001 M $MgCl_2$, and 0.00005 M $MnCl_2$ were also added.

Preparation of rickettsiae. The E strain of epidemic typhus grown in the yolk sac of embryonated eggs (Cox, 1941) was used throughout. The rickettsiae were purified from infected yolk sacs by differential centrifugation as described previously (Bovarnick and Miller, 1950; Bovarnick, 1956), except that the suspending solutions used were varied. In general four types of preparations were used: (I) Those with which sucrose P was used both in harvesting the yolk sacs and throughout the purification procedure; (II) those from yolk sacs collected in sucrose P, but washed at least once with K-7 during the purification; (III) those from yolk sacs collected in K-7 and centrifuged the first time from this salt solution, then purified with sucrose P; (IV) those from yolk sacs collected in K-7 and purified with the same solvent throughout. Bovine serum albumin was used with sucrose and salt media to aid in removal of yolk particles as described in earlier publications (Bovarnick and Miller, 1950; Bovarnick 1956). The final precipitate of purified rickettsiae was resuspended to a concentration corresponding to 4 g original yolk sac per ml, usually in sucrose P, always in this solvent when the rickettsiae were to be stored in the frozen state before use.

Assay methods. Tests for hemolytic activity, respiratory activity, and toxicity for mice were carried out as previously described (Allen *et al.*, 1954). The infectivity for eggs was estimated as described by Golub (1948), except that two

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[2] The following abbreviations will be used: diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; glutathione, GSH; coenzyme A, CoA.

dilutions rather than one were used for each suspension assayed, and the average of the two values for the LD_{50} is given.

Inactivation at 0 C. The concentrated rickettsial suspension was diluted 10- to 40-fold in the desired medium at 0 C and kept at this temperature for the indicated period. If only toxicity, hemolytic activity or infectivity were to be measured, further dilutions were then made for determination of the respective endpoints. In calculating these endpoints the concentration of the suspension kept at 0 C was considered to be unity. If respiratory activity or DPN content were to be measured, the suspension was centrifuged at 5,000 rpm for 45 min, and the rickettsiae were then resuspended to a concentration corresponding to 2 g original infected yolk sac per ml to attain a concentration of rickettsiae sufficient for measurement of these properties. Centrifugation at this point did not materially affect the toxic or hemolytic end points.

Reactivation at 33 C. For reactivation at 33 C rickettsiae that had been kept at 0 C were usually diluted 3-fold into a medium containing 0.0625 M KCl; 0.0625 M NaCl, 0.01 M K phosphate; pH 7.2, 0.005 M glutamate, 0.3 per cent serum albumin, 0.001 M $MgCl_2$, 0.00005 M $MnCl_2$, 0.14 mg DPN per ml, 0.05 mg CoA per ml.; and 0.0007 M GSH, then incubated for 3 hr at 33 C and assayed at the end of this period.

Measurement of DPN content of the rickettsiae. A modification of the method of Huff and Perlzweig (Levitas *et al.*, 1947) was used for these determinations, in which methyl ethyl ketone was substituted for acetone (Burch *et al.*, 1955). A DPN solution whose concentration had been determined by measurement of the absorption at 325 mμ in M KCN (Colowick *et al.*, 1951) was used as standard in these determinations. The method used is of course not specific for DPN alone. In most cells the values found would represent total pyridine nucleotide. In the case of the rickettsiae DPN must represent the major portion of the pyridine nucleotide, since the concentrations found with this method are in the same range as those found earlier with an enzymatic method specific for DPN.

RESULTS

In the first experiments in which the rickettsiae were left overnight in isotonic salt solutions at 0 C, highly variable losses of hemolytic activity and toxicity were observed. It soon became apparent that, among other factors, the method of preparation of the rickettsiae had considerable influence on their stability. Table 1 summarizes the results obtained with all of our preparations in one specific salt solution, chosen because more experiments have been done with this than with any other. In spite of some overlapping between the groups, it is apparent that those preparations kept in sucrose throughout show

TABLE 1

*Loss of toxicity and hemolytic activity of various types of preparations of the E strain of typhus rickettsiae at 0 C**

Type	Method of Preparation	Hemolytic Activity		Toxicity	
		Per cent	Standard deviation†	Per cent	Standard deviation†
I	Sucrose P used throughout	11	8 (10)	8	5 (4)
IF	Same as I, after freezing	9	8 (22)	5	4 (4)
II	Yolk sacs collected in sucrose, washed with sucrose P, K-7, then sucrose P	38	17 (4)	32	14 (4)
III	Yolk sacs collected in K-7, then washed twice with sucrose P	40	16 (6)	25	9 (4)
IIIF	Same as III, after freezing	29	10 (12)	6	(1)
IV	Salt K-7 used throughout	59	16 (2)	25	(1)
IVF	Same as IV, after freezing	41	11 (3)		

* In these experiments the rickettsiae were purified with the use of the indicated media as described more fully in the text, then diluted 10- to 40-fold at 0 C into the basal salt medium with a Na to K ratio of 2 to 1. Toxicity and hemolytic activity were measured initially and after 18 hr at 0 C. The remaining activity is expressed as per cent of the initial activity.

† The figures in parentheses give the number of experiments on which the values are based.

the least stability (type I), those kept in salt, the greatest (type IV). The other two groups, II and III, kept in sucrose throughout most of the purification procedure but treated with salt at one stage, are indistinguishable in their behavior and intermediate between the other two types. After freezing, which was always carried out in the presence of sucrose, there was a decrease in the stability of all types of preparations.

The composition of the medium in which the rickettsiae are diluted also affects the stability of all types of preparations at 0 C, the factors of importance being the presence or absence of DPN, Mg, Mn, Na, and glutamate, and the pH of the medium (table 2). Addition of Mg and Mn or of DPN, or an increase in pH from 7.0 to 7.3, each leads to an increase in stability, although no single one of these factors can completely prevent loss in activity at 0 C.

As can be seen in table 2, the effects of glutamate and sodium are mutually interdependent. When the sodium concentration is high, addition of glutamate leads to marked decrease in stability at 0 C. On the other hand, glutamate in the absence of sodium, or sodium in the absence of glutamate, each has little influence on the stability at 0 C. It should be noted that this inhibitory effect of glutamate is quite specific. It is not found with other substrates of the rickettsiae, such as pyruvate or succinate, nor with any other amino acid.

In all cases the loss in toxicity and hemolytic activity that occurred at 0 C could be largely reversed by subsequent incubation at 33 C in a medium containing DPN, CoA, Mg, Mn, and glutamate and albumin. After such incubation 60 to 100 per cent of the initial activity was regularly restored (table 3). In the absence of DPN reactivation was never possible and no coenzyme other than DPN appeared to be re-

TABLE 2

*Influence of the composition of the medium on the loss of toxicity and hemolytic activity of the E strain of typhus rickettsiae at 0 C**

Type of Preparation	Conditions of Incubation at 0 C						Toxicity LD_{50}†		Hemolytic Activity HE‡	
	Na/Na + K	pH	Mg and Mn	Glutamate	Diphosphopyridine nucleotide	Coenzyme A	Initial	After 0 C inactivation	Initial	After 0 C inactivation
I	0.67	7.0					16	1.2	60	3.8
	0.67	7.0			+	+	16	1.4		6.9
	0.67	7.0		+			16	<0.7		0.5
	0.67	7.0		+	+	+	16	2.1		9.4
	0.67	7.3	+	+	+	+	16	14		45
IF	0.67	7.0					17	<0.7	61	4
	0.67	7.0	+				17	2.3		20
	0.67	7.3					17	0.7		13
	0.67	7.3	+				17	5.5		24
	0.67	7.0			+		17	2.3		17
III	0.0	7.0					17	5.5	90	49
	0.0	7.0		+			17	5.5		39
	0.5	7.0					17	4.5		54
	0.5	7.0		+			17	1.4		10
	1.0	7.0					17	5.5		49
	1.0	7.0		+			17	<0.7		7

* These experiments were carried out in the same way as those in table 1, except that the pH of the phosphate was varied and $MgCl_2$, $MnCl_2$, glutamate, or diphosphopyridine nucleotide (DPN) was added as indicated, using the concentrations given in the text.

† The LD_{50} represents the denominator of the dilution required to kill 50 per cent of the mice.

‡ The HE represents the denominator of the dilution required to give an optical density reading of 0.3 in the hemolysin assay method (Snyder *et al.*, 1954).

TABLE 3

*Reactivation of the toxicity and hemolytic activity of typhus rickettsiae after inactivation at 0 C**

Assay Method	Initial Titer	Titer After 18 Hr at 0 C	Reactivation at 33 C Additions to Basal Medium			
			Diphosphopyridine nucleotide	Coenzyme A	Mg and Mn	Titer After 3 Hr
Toxicity†	17	0.7	+	+		13
			+	+	+	16
			+		+	16
				+	+	0.7
Hemolytic activity†	60	0.5	+	+		55
			+		+	60
			+	+	+	70
				+	+	1.0

* These experiments were done with type I preparations, diluted at 0 C in the basal medium used in table 1. The reactivation was carried out as described in the text, except that some of the constituents were omitted from the reactivation medium as indicated.

† The significance of the figures given for toxic and hemolytic activities is as given in the footnote to table 2.

TABLE 4

*Reversible loss of infectivity for eggs of typhus rickettsiae at 0 C**

Treatment of Rickettsiae	$-\text{Log } LD_{50}$	$\Delta\text{Log } LD_{50}$	Per Cent of Infectivity Remaining
A. None	7.4		100
B. 18 hr at 0 C	5.3	2.1	0.8
C. 18 hr at 0 C with glutamate	4.6	2.8	0.2
D. B, incubated 3 hr at 33 C with coenzyme A (CoA) and diphosphopyridine nucleotide (DPN)	7.2	0.2	63
E. C, incubated 3 hr at 33 C with DPN and CoA	6.8	0.6	25

* This experiment was done with a type IF preparation. The rickettsiae were diluted 40-fold into the basal medium used in table 1, with addition to C of 0.005 M glutamate. In addition to DPN and CoA, $MgCl_2$ and $MnCl_2$ were added to D and E, and glutamate to D, in the usual concentrations.

quired for reactivation. TPN could not replace DPN. Mg, Mn, and CoA, although usually added, contributed only slightly to the degree of reactivation. The function of glutamate cannot be evaluated since it is required even by un-

TABLE 5

*Oxidative activity of typhus rickettsiae kept for 18 hr at 0 C**

Type	Medium at 0 C				Rate of Oxygen Uptake†	
	Na/Na + K	Glutamate	Mg and Mn	pH	Without coenzymes	With DPN and CoA‡
					μL/hr	*μL/hr*
I	Control				64	74.5
	0.67			7.0	0.7	57
	0.67	+		7.0	0.0	55
	0.00			7.0	2.0	61
	0.00	+		7.0	4.0	52
	0.67		+	7.3	14	61
	0.67	+	+	7.3	5	63
	0.00		+	7.3	15	63
	0.00	+	+	7.3	10	62
III	Control				71	91
	0.67			7.0	17	72
	0.67	+		7.0	3	49
	0.0			7.0	21	75
	0.0	+		7.0	26	72
	0.67		+	7.3	27	70
	0.67	+	+	7.3	11	69
	0.0	−	+	7.3	36	70
	0.0	+	+	7.3	26	69

* The rickettsiae were diluted in the basal medium described in the experimental section, the sodium to potassium ratio and the pH being varied, and glutamate, $MnCl_2$, $MgCl_2$, were added as indicated. The diluted suspensions were left for 18 hr at 0 C, then centrifuged at 0 C. The rickettsiae were resuspended to a concentration corresponding to 2 g original infected yolk sac per ml in the all-potassium basal medium for measurement of respiratory activity. The respiratory activity of the controls was measured initially, without dilution or centrifugation.

† For the oxygen uptake measurements each vessel contained the following, in μmoles: KCl, 109; potassium phosphate, pH 7.5, 15; $MgCl_2$, 1.5; $MnCl_2$, 0.08; K glutamate, 16; and 0.4 ml of the rickettsial suspension in a total volume of 1.5 ml. DPN, 0.27 mg; CoA, 0.1 mg; and GSH,‡ 1 μmole, were added when indicated.

‡ DPN = diphosphopyridine nucleotide; CoA = coenzyme A; GSH = glutathione.

damaged rickettsiae at 33 C (Bovarnick *et al.*, 1950). It seemed somewhat surprising that Mg and Mn contributed so little to the reactivation, since their stabilizing effect at 0 C suggested that these ions, like DPN, might be lost at the lower temperature.

The changes in toxicity and hemolytic activity were paralleled by similar changes in infectivity and respiratory activity (tables 4 and 5).

As anticipated from the fact that DPN was specifically required for reactivation, it was found that this coenzyme is lost from rickettsiae kept at 0 C (table 6). Qualitatively the loss in DPN parallels the loss of other properties and varies in the same way with the type of preparation and the composition of the medium. Quantitatively, however, the agreement is not good. The percentage loss in DPN is usually considerably less than the percentage loss in hemolytic activity or toxicity. This discrepancy, considered together with the fact that DPN added at 0 C does not completely prevent the drop in activity, suggests that loss of DPN may not be the sole cause of the loss of activity.

TABLE 6

*Diphosphopyridine nucleotide (DPN) content of typhus rickettsiae kept at 0 C**

Type	Treatment of Rickettsiae at 0 C				DPN Content		Hemolytic Activity
	Time	Additions to basal medium					
		Glutamate	Mg and Mn	pH			
	hr				*μg*	*% initial*	*% initial*
IF	0				2.20	100	100
	3	−	−	7.0	0.49	22	5
	3	−	−	7.3	0.62	28	19
	3	−	+	7.3	0.74	34	49
	3(33 C)	+	−	7.0	1.09	50	46
IIIF	0				2.65	100	100
	4	−	−	7.0	1.19	45	20
	4	+	−	7.0	0.73	28	6
IF	0	−	−		1.94	100	100
	18	−	−	7.0	0.22	9	2
	18	+	−	7.0	0.22	9	<1

* The basal medium was the same as that used in table 1. Mg, Mn, and glutamate when present were added in the usual concentrations. The rickettsiae were diluted and left for the indicated period at 0 C, then centrifuged at 0 C, and resuspended to a concentration corresponding to 2 g original infected yolk per ml before assaying for DPN content and hemolytic activity. In one instance, as indicated, the suspension was left at 33 C rather than at 0 C.

TABLE 7

*Comparative rate of loss of toxicity and hemolytic activity by typhus rickettsiae at 0 C and at higher temperatures**

Type	Na/Na+K	Time of Incubation	Toxicity† Remaining After Incubation at:		Hemolytic† Activity After Incubation at	
			0 C	33 C	0 C	33 C
		hr	%	%	%	%
IF	0.0	4			28	43
	0.67	4			6	35
IF	0.50	18‡	<7	55	4	57
IF	0.50	18‡	<5	10	<1	29
IIF	0.67	4	10	32	11	56
IIIF	0.67	4			33	56

* In these experiments 0.005 M glutamate was included in the basal medium in all cases and the pH was 7.0.

† The figures for toxicity and hemolytic activity represent per cent of the initial activity remaining.

‡ In these experiments the higher temperature was 27 C rather than 33 C.

Table 6 also shows that DPN is retained in the rickettsiae better at 33 C than at 0 C. This is consistent with the behavior of the toxicity and hemolytic activity of preparations of rickettsiae kept at the two temperatures, as shown in table 7. In the presence of glutamate these two properties of the rickettsiae are also more stable at 33 C than at 0 C, especially in media of high Na concentration. The relatively greater stability at the higher temperature does not hold true in the absence of substrate.

DISCUSSION

It is difficult to offer any fully adequate explanation of the results described above. It is apparent that certain types of rickettsiae, those kept most completely in sucrose throughout their purification, quite rapidly lose activity to 0 C when diluted in an isotonic salt solution. They simultaneously lose some of their DPN and the loss in activity is largely reversible on subsequent incubation with DPN. However, since the decrease in activity is more rapid than the loss of DPN and particularly since DPN added at 0 C can only lessen, not prevent loss of activity, leakage of this coenzyme cannot be the sole cause

of the change in activity. On the other hand no other coenzyme is needed for restoration of activity at 33 C. These facts, as well as the stabilizing effect of Mg and Mn, suggest that at 0 C, where metabolic activity is low, rickettsiae, like other cells, have difficulty in maintaining the normal internal concentrations of a variety of diffusible constituents, including certain inorganic ions as well as DPN. Loss of each of these substances may contribute to the loss in activity observed at 0 C. The adverse effects of Na and glutamate together might then be due to the fact that Na and K are transferred into and out of the rickettsiae most readily as their glutamate salts, thus leading to replacement of cellular K by Na under these conditions. The great sensitivity of preparations made entirely with sucrose to dilution in salt solutions at 0 C may be due to uptake of some sucrose by such preparations, followed by exchange of sucrose for salt on dilution into a nonsucrose containing medium. At 0 C the metabolic activity does not suffice to control the exchange with the result that it is accompanied by loss of other constituents of the cell, certainly of DPN, possibly also of Mg and Mn.

It may be significant that each of the factors that tend to increase the stability of the rickettsiae at 0 C—namely Mg, Mn, increase in pH, and DPN—also is known to increase the rate of oxygen consumption at 34 C (Bovarnick and Snyder, 1949; Wisseman *et al.*, 1951; Bovarnick *et al.*, 1953). Evidence of a similar effect of these factors on the metabolic rate at 0 C can be obtained only with difficulty due to its very low value at this temperature. The rickettsiae utilize glutamate at 0 C at a rate 1 to 1½ per cent of the rate observed at 34 C, and this rate is apparently increased by the same factors that increase the rate at 34 C. Any such stimulation of the very low rate of metabolic activity of the rickettsiae at 0 C should increase their ability to prevent changes in the concentration of internal diffusible constituents and should therefore also increase their stability. Since the addition of substrate rarely increases their stability at 0 C, the suggestion that the medium exerts its influence on the stability of the rickettsiae in part through its effect on their metabolic rate presumes that these organisms have some endogenous metabolism. It is true that endogenous oxygen uptake has not been observed, but an oxygen uptake equivalent to the amount of glutamate consumed by these organisms in 18 hr at 0 C would be taken up in 15 to 20 min at 34 C, which is the period of time required for temperature equilibration in measurements of oxygen uptake. Such a quantity of endogenous substrate could therefore pass undetected at 34 C, yet suffice for maintenance of rickettsiae for a relatively long period at 0 C.

A corollary to the assumption that at 0 C the rate of metabolic activity is a limiting factor in the maintenance of normal concentrations of the diffusible constituents of the rickettsiae is the further assumption that at higher temperatures, where their metabolic rate is much more rapid, they, like other cells (Maizels, 1954) can maintain, or if necessary restore to normal, their characteristic internal environment, even against unfavorable concentration gradients in the external medium. Such an assumption is supported by the fact that the concentration of various salts in the medium is less critical for maintenance and restoration of activity at 33 C than at 0 C and by the observation that both DPN and activity are lost less rapidly at the higher temperature.

This suggested explanation for the results described herein is still of course largely hypothesis. Actual measurement of the electrolyte content of rickettsiae under different conditions should indicate whether in fact changes in the concentration of inorganic ions as well as of DPN do take place in the rickettsiae and can bring about alterations in their biological activity.

SUMMARY

Purified preparations of the E strain of typhus rickettsiae lose toxicity, hemolytic activity, respiratory activity, and infectivity for eggs when diluted in isotonic salt solution at 0 C. The rate of loss is affected by many factors, including the media used in purification of the rickettsiae and the composition of the solution used for dilution. The greatest losses are exhibited by rickettsial preparations with which sucrose solutions were used for the entire purification procedure, the least with those prepared with isotonic salt solution. With all types of preparations, stability at 0 C is increased by addition of Mg and Mn or DPN or by increasing the pH from 7 to 7.3 and is decreased by glutamate in the presence of high concentrations of Na (but not in an all K

medium). Loss in activity at 0 C is accompanied by loss in DPN content.

In every instance 50 to 100 per cent of the initial activity of the rickettsiae inactivated at 0 C could be restored by subsequent incubation at 33 C in the presence of DPN.

Since the presence of DPN at 0 C cannot prevent the loss in activity under all conditions, loss of this substance alone cannot account for all of the changes observed. It is suggested that changes in the concentration of certain inorganic ions in the rickettsiae may also contribute to the loss in activity.

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