THE INFLUENCE OF DIPHOSPHOPYRIDINE NUCLEOTIDE ON THE STABILITY OF TYPHUS RICKETTSIAE[1]

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A study of the factors influencing the stability of typhus rickettsiae *in vitro*, initiated several years ago (Bovarnick *et al.*, 1950), has been extended with the aim of ultimately achieving growth *in vitro* of these organisms. During these experiments it was noted that survival was increased consistently by addition of diphosphopyridine nucleotide (DPN) to the basal medium. In addition, diphosphopyridine nucleotide increased the rate of oxygen uptake by the rickettsiae with glutamate as substrate. Since both of these effects were relatively small and the diphosphopyridine nucleotide preparations were not pure, it was thought that an impurity might be responsible for the result. Therefore, a sample of diphosphopyridine nucleotide that had been autoclaved to destroy the diphosphopyridine nucleotide itself was tested. Not only was the stimulatory effect removed by this procedure, but the autoclaved diphosphopyridine nucleotide proved to be strongly inhibitory to the survival of the rickettsiae and partially so to their rate of oxygen uptake. This inhibition could be prevented completely by simultaneous addition of untreated diphosphopyridine nucleotide. It is believed that these results indicate that diphosphopyridine nucleotide, or possibly some impurity of equal heat lability, is of considerable importance to the metabolism of the rickettsiae.

EXPERIMENTAL METHODS

Rickettsiae. The Madrid E strain of typhus rickettsiae, grown in the yolk sacs of embryonated eggs, washed, and concentrated as previously described (Bovarnick and Miller, 1950) was used. For the survival experiments the rickettsiae were washed only once; for the oxygen uptake experiments they were purified further by treatment with albumin.

Methods of assay. Mouse toxicity. Groups of four mice were injected intravenously with 0.25 ml of serial threefold dilutions of the suspensions of rickettsiae (Bengston *et al.*, 1945). Deaths were counted after 24 hours, and the dilution required to kill 50 per cent of the mice was estimated by the method of Reed and Muench (1938).

Infectivity for eggs. Relative numbers of surviving rickettsiae were estimated as described in a previous publication (Bovarnick *et al.*, 1950) by inoculation of groups of 12 seven day fertile eggs by the yolk sac route with 0.2 ml of suspensions of rickettsiae. The eggs were candled daily for 11 days after inoculation, and the average time of death of eggs dying after the third day was calculated. An increase of one day in the average day of death corresponds approximately to a tenfold decrease in the number of viable rickettsiae with this strain.

Hemolytic activity. This was measured by the method of Clarke and Fox (1948) as modified by Snyder *et al.* (*to be published*). In brief, sheep red cells were washed three times and resuspended to make a 25 per cent suspension in the diluent described below. A 0.2 ml quantity of serial threefold dilutions of the rickettsiae to be assayed was mixed with 0.4 ml of the sheep cell suspension in optically matched Kahn tubes and incubated at 34 C for 2½ hours with occasional shaking. Then 2.0 ml saline containing 0.1 per cent formalin was added to each tube, and the contents were mixed and centrifuged at 2,000 rpm for 15 minutes. The optical density was read then at a wavelength of 545 mμ in a Coleman Junior spectrophotometer, using as a blank a tube containing diluent in place of rickettsiae. The optical density reading is a linear function of the concentration of rickettsiae up to an optical density of about 0.6. An optical density of 0.3 was chosen arbitrarily as the hemolytic end point,

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and the concentration required to produce this reading was estimated graphically from the observed values.

The diluent used in the hemolysin assay varied with the medium used in the survival experiments. When the rickettsiae were incubated in a sucrose medium, the sheep cells were washed and suspended and dilutions of rickettsiae were made in 0.225 M sucrose containing 0.01 M potassium phosphate, pH 7, 0.005 M potassium glutamate, and 0.005 M $MgCl_2$. For experiments in the salt media the diluent contained 0.089 M NaCl, 0.042 M KCl, 0.01 M potassium phosphate, pH 7, and 0.01 M potassium glutamate. The red cells were washed and suspended in this medium with added $MgCl_2$, 0.007 M; $CaCl_2$, 1.5×10^{-4} M; and $MnCl_2$, 1.5×10^{-4} M. Dilutions of rickettsiae were made in the metal free salt solution to which was added 0.5 per cent normal yolk sac that had been heated at 56 C for 30 minutes.

Oxygen consumption. This was measured by the usual Warburg technique.

Media. Two types of media were used in the survival experiments: one made isotonic with sucrose, the other with KCl. Both contained per liter: Casein hydrolyzate, 0.72 g; glutamic acid, 0.48 g; tryptophan, 19 mg; $MgCl_2$, 1.44 mm; $MnCl_2$, 0.077 mm; $CaCl_2$, 0.019 mm; K_2HPO_4, 2.6 mm; KH_2PO_4, 5 mm; $NaHCO_3$, 3.4 mm, and were adjusted to pH 7 with KOH. One medium containing in addition to the above, sucrose, 0.181 M, will be referred to as the sucrose medium. The other containing KCl, 0.113 M, in place of the sucrose will be referred to as the salt medium. In some instances a mixture of all available vitamins also was added, but this had no observable influence on the survival.

The diphosphopyridine nucleotide samples used were commercial preparations of varying degrees of purity. There was no marked difference in the effects observed with any of the samples. The diphosphopyridine nucleotide was made up to a concentration of 5.4 mg per ml, adjusted to approximately pH 6.8, and sterilized by filtration through an U.F. glass filter. Autoclaved diphosphopyridine nucleotide was made up similarly, then autoclaved at 120 C for 7 minutes.

Immediately before use, a freshly prepared, neutralized solution of glutathione in a final concentration of 0.0007 M and Armour's crystalline bovine albumin, 0.2 per cent, were added to the above media. At the same time, diphosphopyridine nucleotide untreated or autoclaved was added where indicated.

Survival experiments. When hemolytic activity or toxicity for mice was used as the method of assay, the rickettsiae were added to the medium to be tested in a concentration equivalent to 10 per cent original yolk sac. When infectivity for eggs was used as the method of assay, the concentration of rickettsiae was equivalent to 0.1 per cent original infected yolk sac. In all cases the mixtures were assayed before incubation and again after incubation at 34 C for 16 hours. Preliminary experiments in which the rickettsiae were assayed before incubation with and without addition of diphosphopyridine nucleotide or autoclaved diphosphopyridine nucleotide showed that the values found initially were independent of the added substances within the limits of error of the methods used (table 1). Therefore, in most of the survival experiments only one or two mixtures were assayed before incubation.

TABLE 1

Hemolytic activity, toxicity, and infectivity of rickettsiae measured immediately after mixing with diphosphopyridine nucleotide (DPN) or autoclaved diphosphopyridine nucleotide

METHOD OF ASSAY	SUBSTANCE ADDED		
		DPN	Autoclaved DPN
Hemolysin end point*	5.6	5.6	5.6
	6.0	5.7	5.9
	12.0	11.2	11.8
Mouse toxin, LD_{50}†	5.5	5.5	—
	2.3	2.1	2.1
Egg infectivity, day of death	5.8	6.1	—

* The figures represent the denominator of the dilution of the mixture that caused sufficient hemolysis to give an optical density of 0.3 in the hemolysin assay.

† The LD_{50} is expressed as the denominator of the dilution required to kill 50 per cent of the mice. The salt containing basal medium was used in the first hemolysin experiment and in the egg infectivity experiment; the sucrose basal medium, in the others.

RESULTS

The influence of diphosphopyridine nucleotide, autoclaved diphosphopyridine nucleotide, and mixtures of both on the survival of rickettsiae as measured by their hemolytic activity, toxicity for mice, or infectivity for eggs is shown in tables 2, 3, and 4. The survival in all cases is increased by the presence of diphosphopyridine nucleotide, decreased by autoclaved diphosphopyridine nucleotide alone, but unaffected by autoclaved diphosphopyridine nucleotide in the presence of native diphosphopyridine nucleotide, except in those few instances where survival in the controls was either so good as to make any improvement insignificant, or so poor as to make any decrease undetectable. While the relative influence of diphosphopyridine nucleotide is quite reproducible, it is apparent that the absolute survival as measured by our methods is variable. The factors causing this variability are still unknown.

The influence of diphosphopyridine nucleotide and autoclaved diphosphopyridine nucleotide

TABLE 2

Effect of diphosphopyridine nucleotide (DPN) and autoclaved diphosphopyridine nucleotide on the survival of rickettsiae at 34 C as measured by the hemolysin method

		INCUBATION PERIOD				
		None	16 hours at 34 C			
		Substance added to basal medium				
			None	DPN	Autoclaved DPN	DPN and autoclaved DPN
Basal medium	DPN preparation*					
Salt medium	S-65	3.9	2.9	3.3		
	S-85			3.3		
	S-65	8.3	0.7	1.8		
	S-85			1.9		
	S-65	14.7	3.7	7.1	1.3	7.7
	Σ-65	7.9	1.0	3.1	<0.3	3.0
	Σ-65	6.5	2.2	4.2	0.5	3.6
	Σ-90	4.8	1.1		<0.3	4.0
Sucrose medium	Σ-90	5.8	3.3	4.4	<0.3	3.5
	S-85	6.0	4.5	5.3	0.7	6.3
	Σ-90	6.4	6.4	6.8	0.3	6.8

The figures represent the denominator of the dilution of the mixtures that cause sufficient hemolysis to give an optical density reading of 0.3 in the hemolysin assay. Values of less than one for the end point are estimated from the readings obtained with the undiluted mixtures, assuming a linear relation between optical density reading and concentration.

* The significance of the abbreviations used to designate the source and purity of the diphosphopyridine nucleotide preparations used is as follows: S-65 and S-85, diphosphopyridine nucleotide obtained from the Schwarz Laboratories, stated to be of 65 per cent and 85 per cent purity, respectively; Σ-65 and Σ-90, diphosphopyridine nucleotide obtained from the Sigma Chemical Company, stated to be of 65 per cent and 90 per cent purity, respectively. The final concentration for the 65 per cent preparations was 0.35 mg per ml; for the 90 per cent preparations, 0.27 mg per ml.

on the rate of oxygen uptake by rickettsiae with glutamate as substrate is shown in table 5. These effects are smaller in degree than those on

TABLE 3

Effect of diphosphopyridine nucleotide (DPN) and autoclaved diphosphopyridine nucleotide on the survival of rickettsiae as measured by their infectivity for embryonated eggs

INCUBATION PERIOD			
None	16 hours at 34 C		
Substance added to basal medium			
	None	DPN	DPN, autoclaved
	Average day of death of eggs		
6.0	7.9	6.7	
6.1	8.2	7.6	11 (10/11)*
6.6	7.6		10 (7/11)

* The figures in parentheses give the number of eggs alive on the eleventh day after inoculation divided by the total number of eggs alive on the third day after inoculation. In all of these experiments the salt medium was used.

TABLE 4

Effect of diphosphopyridine nucleotide (DPN) and autoclaved diphosphopyridine nucleotide on the survival of rickettsiae as estimated by their toxicity for mice

INCUBATION PERIOD AND TEMP	INITIAL TITER	TITER AFTER INCUBATION IN THE PRESENCE OF			
			DPN	DPN, auto.	DPN + DPN auto.
	LD_{50}*	LD_{50}	LD_{50}	LD_{50}	LD_{50}
16 hr, 33 C	5.5	5.5		0.7	5.5
18 hr, 33 C	5.5	<0.7	1.4	<0.7	0.7
12 hr, 33 C	5.5	1.7	2.8	<0.7	3.0
5 hr, 36 C	4.5	1.7	2.1	<0.7	3.0

* The LD_{50} is expressed as the denominator of the dilution of the mixture required to kill 50 per cent of the mice. In all of these experiments the sucrose medium was used. Diphosphopyridine nucleotide, obtained from the Sigma Chemical Company and stated to be 90 per cent pure, was used in the above experiments at a concentration of 0.27 mg per ml.

survival but are similar in direction and equally consistent. The effect on O_2 uptake is not progressive as the rates are fairly constant under all conditions for about 3 hours. Therefore, it is probable that the decrease in rate of oxygen uptake brought about by autoclaved diphosphopyridine nucleotide is not a secondary result of the lowered survival of rickettsiae in its presence, but rather due to an inhibitory effect on some reaction directly involved in the oxidation. The effects of autoclaved diphosphopyridine nucleotide on either survival or rate of oxygen uptake cannot be reproduced by autoclaved adenine, adenosine, adenosine-5-phosphate, or adenosine diphosphate with or without addition of nicotinamide. The nature of the toxic substance produced is still under investigation.

TABLE 5

Effect of diphosphopyridine nucleotide (DPN) and autoclaved diphosphopyridine nucleotide on the rate of oxygen uptake by rickettsiae

DPN PREPARATION	SUBSTANCE ADDED			
	None	DPN	Autoclaved DPN	DPN and Autoclaved DPN
	µL per hr	*µL per hr*	*µL per hr*	*µL per hr*
Σ-65, 0.59 mg/ml	32	48	20	
S-65, 0.59 mg/ml	32	48	24	
Σ-90, 0.45 mg/ml	59	68	34	58
Σ-90, 0.45 mg/ml	36	47	12	25
S-85, 0.36 mg/ml	21	36	13	37

Each flask contained glutamate, 0.014 M, $MgCl_2$, 0.002 M, $MnCl_2$, 0.00033 M, potassium phosphate, 0.01 M, pH 7.3, KCl, 0.12 M and 1 ml of a rickettsiae suspension whose concentration was equivalent to 200 per cent original infected yolk sac. Control experiments indicated that in the absence of glutamate or with similarly treated preparations of normal yolk sac substituted for the rickettsiae, diphosphopyridine nucleotide brought about no significant oxygen uptake. For the significance of the abbreviations used in column 1, see table 2, footnote.

The apparent importance of diphosphopyridine nucleotide to the metabolism of the rickettsiae naturally suggests the possibility that other coenzymes also may be required by these organisms. Thus far, of those available in relatively pure form, adenosine triphosphate, adenylic acid, cocarboxylase, pyridoxamine phosphate, and coenzyme A (Pabst) appear to be without marked effect; flavin adenine dinucleotide and riboflavin phosphate appear to be slightly toxic; triphospho-

pyridine nucleotide (10 per cent, Sigma Chemical Co.) behaves in the same way as diphosphopyridine nucleotide, but further investigation with a more highly purified preparation will be required to demonstrate whether the two are really interchangeable. Crude coenzyme preparations, such as Armour's coenzyme concentrate, or hot water extracts of yeast or liver are usually markedly toxic. The results with autoclaved diphosphopyridine nucleotide suggest that this toxicity might be due in part to certain coenzyme decomposition products in the crude preparations and extracts.

SUMMARY

The survival of rickettsiae as measured by their toxicity for mice, hemolytic activity, or infectivity for eggs is increased in the presence of added diphosphopyridine nucleotide and markedly decreased by autoclaved diphosphopyridine nucleotide. The toxic effect of autoclaved diphosphopyridine nucleotide can be prevented by the simultaneous addition of unaltered diphosphopyridine nucleotide.

Diphosphopyridine nucleotide increases the rate of oxygen uptake of rickettsiae with glutamate as substrate. Autoclaved diphosphopyridine nucleotide at a concentration of 0.27 mg per ml inhibits the rate of oxygen uptake 40 to 50 per cent. This inhibition can be prevented by addition of unaltered diphosphopyridine nucleotide.

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