REVERSIBLE INACTIVATION OF TYPHUS RICKETTSIAE*

I. Inactivation by Freezing

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(Received for publication, June 9, 1954)

During experiments designed to test the effect of a variety of media on the stability or possible extracellular growth of the Madrid E strain of typhus rickettsiae, occasional increases in hemolytic titre were noted. These increases were small and unpredictable, but beyond the limits of error of the assay method. The rickettsiae used in these experiments had been partially purified, suspended in isotonic sucrose, frozen, and stored at −80°C. until used. It gradually became apparent that the increases took place with suspensions of rickettsiae whose initial titres were lower than would have been anticipated from other preparations made from the same starting material. This led to the hypothesis that the apparent increases might actually represent restoration of activity to partially damaged organisms, and it seemed possible that the damage had taken place during the process of freezing and thawing. Since in the presence of isotonic sucrose loss of activity on freezing is usually slight (1), more severely damaged rickettsiae were obtained by freezing and thawing in an isotonic salt solution, a process known to lead regularly to marked inactivation (1). Rickettsiae treated in this way did indeed have greatly reduced hemolytic activity, toxicity, and infectivity. However, when such organisms were incubated in a suitable medium, of which diphosphopyridine nucleotide (DPN) was the most important constituent, for 2 to 3 hours at 34°C. there was a marked increase, often as much as tenfold, in hemolytic activity, toxicity, and respiratory activity. In several experiments there also appeared to be a comparable increase in the infectivity for eggs. In most cases only a fraction of the initial activity could be restored. This was somewhat increased by addition of coenzyme A (CoA) as well as DPN. Whether addition of other cofactors will lead to more complete restoration of activity remains to be determined.

It should be stated that in the following discussion the terms inactivation

* This work was supported by a research grant from the Division of Research Grants of the National Institutes of Health, United States Public Health Service (E167C2).

and reactivation are used in a purely descriptive sense. Inactivated rickettsiae are considered to be organisms that no longer demonstrate hemolytic activity, toxicity, or infectivity under the usual assay conditions; reactivated rickettsiae are those which have lost, then regained these properties after suitable treatment.

Methods

Materials.—The DPN used was obtained from the Sigma Chemical Company and was stated to be 90 per cent pure. CoA was obtained from the Pabst Laboratories. Fructose diphosphate (HDP) was obtained as the barium salt from the Schwarz Laboratories and was converted to the potassium salt with K_2SO_4 before use. Crystalline triosephosphate dehydrogenase was prepared by the method of Cori, Slein, and Cori (2) and was freed from DPN immediately before use as described by Velick *et al.* (3). Crystalline aldolase was prepared by the method of Taylor *et al.* (4). Alcohol dehydrogenase was prepared as described by Racker (5).

The isotonic salt and sucrose solutions, referred to as K-7G and sucrose PG respectively, both of which contained 0.01 M phosphate buffer, pH 7, and 0.005 M glutamate have been previously described (1). These were used in washing the rickettsiae and in preparing dilutions for assays.

The basal medium used in the reactivation experiments contained casein hydrolysate, phosphate, bicarbonate, trace metals, and has been described by Allen *et al.* (6). In most experiments heated normal yolk sac was also added to the basal medium. For this purpose yolk sacs were collected from 13 day embryonated eggs and were homogenized with 1 volume of K-7G, then heated for 30 minutes at 56°C.; 0.01 volume of this 50 per cent suspension was added to the basal medium to improve the stability of the rickettsiae.

Rickettsiae.—The Madrid E strain of typhus rickettsiae, grown in the yolk sac of embryonated eggs, was washed once with sucrose PG solution as previously described (6). The rickettsial precipitate from the sucrose medium was washed once with the isotonic salt solution K-7G and was resuspended in K-7G containing the indicated amount of sucrose to a concentration corresponding to 2 gm. per ml. of original infected yolk sac. This suspension was assayed at once, then frozen in a dry ice alcohol bath and thawed at 34°C. once or twice as indicated.

Assay Methods.—Tests for hemolytic activity, toxicity for mice, infectivity for eggs, and respiratory activity were carried out as previously described (6).

Reactivation Experiments.—Samples of rickettsiae that had been frozen in isotonic salt solution were added to the chilled test medium using 0.5 to 1.0 volume of rickettsiae per 10 volumes of final suspension. DPN and CoA were also added where indicated to a final concentration of 0.14 mg./ml. and 0.05 mg./ml. respectively. Whenever CoA was used glutathione (GSH) was also added in a final concentration of 0.0007 M. Samples were removed for determination of hemolytic, toxic, or infectivity titres immediately after the mixtures were prepared. The mixtures were then incubated for 2 to 3 hours at 34°C., and again assayed.

Measurement of the DPN Content of the Rickettsiae.—This was assayed enzymatically on trichloracetic acid extracts of the organisms, using a system containing HDP, acetaldehyde, aldolase, triosephosphate dehydrogenase, alcohol dehydrogenase, ar-

senate, bicarbonate, and CO_2. The rate of CO_2 evolution in the presence of high concentrations of substrates and enzymes is dependent upon the concentration of DPN added. For preparation of the extracts 2 ml. or more of rickettsial suspension was treated at 0°C. with 0.1 volume of 100 per cent trichloracetic acid (TCA). After centrifugation, 0.1 volume of 0.2 N HCl was added to the TCA extract and this was then extracted 8 times with ether. The remaining aqueous solution was neutralized with KOH, aerated to remove the ether, and 1 ml. aliquots were used for the DPN analysis. In some instances the original rickettsial suspension was first centrifuged at 0°C. for 45 minutes at 5000 R.P.M. The precipitate containing the rickettsiae was resuspended to the original volume and rickettsiae and supernatant were separately treated with TCA and analyzed for DPN.

The measurement of the above mentioned enzymatic reaction was done by the usual Warburg manometric method. Each flask contained in the main compartment the following constituents (in micromols): HDP, 30; acetaldehyde, 60; sodium arsenate, 18; $NaHCO_3$, 150; GSH, 2. The desired quantity of DPN, 0.15 to 1.5 μg., or of rickettsial extract, and water to bring the volume to 2.8 ml. were also added. The side arm contained 180 μg. aldolase, 270 μg. triosephosphate dehydrogenase, 10 micromols $NaHCO_3$, and 0.8 micromol GSH, dissolved in 0.2 ml. of 0.01 M phosphate, pH 7.5. After aeration for 7 minutes with a 4 per cent CO_2-air mixture that had been passed through 0.06 M acetaldehyde to avoid loss of acetaldehyde, the flasks were shaken for 10 minutes, the contents then mixed, and readings were taken at 5 to 10 minute intervals for 40 to 50 minutes. The microliters of CO_2 evolved in the 30 minute period between 10 and 40 minutes after mixing were plotted against the amount of DPN present in the controls for each experiment and from this curve the amount of DPN in the unknown samples could be estimated. Control experiments indicated that TCA extracts of particles that had been prepared from normal yolk sacs by the same procedure as was used for preparation of rickettsiae from infected yolk sacs, not only gave no greater rate of CO_2 evolution than the low rate (3 to 6 microliters per 30 minutes) observed in the absence of DPN, but actually decreased the response obtained with added DPN. This decrease in rate did not appear to be due to residual TCA or ether, since addition of similarly treated extracts of serum albumin solution to the complete system showed no such inhibition. The average decrease noted in several experiments was 25 per cent. Applying this correction, the recovery of DPN added to normal yolk particles before TCA precipitation was 90 per cent. This is probably within the error of the assay, since the small volumes of material available and the necessity for avoiding any unnecessary dilution precluded precise estimation of the dilution factor after addition of TCA, HCl, ether extraction, and neutralization.

RESULTS

A typical experiment illustrating the drop in hemolytic and toxic titres, and in respiration after rickettsiae are frozen in the presence of low concentrations of sucrose and the subsequent recovery on incubation in the presence of DPN and CoA is given in Table I. It can be seen that the extent of both the drop and the recovery depends upon the concentration of sucrose when the rickettsiae are frozen and thawed. In the presence of 0.044 M sucrose, 75 to 80 per

cent of the hemolytic activity and 50 per cent of the toxicity remain after freezing. In the complete absence of sucrose, there is no detectable activity after freezing, and recovery after treatment with coenzymes amounts to not more than 10 to 30 per cent of the initial activity. For most experiments 0.0029

TABLE I

Changes in the Toxic, Hemolytic, and Respiratory Activity of Suspensions of Typhus Rickettsiae after Freezing and Thawing in the Presence of Varying Concentrations of Sucrose, Followed by Incubation with DPN and CoA

Method of assay	Treatment of rickettsiae	Concentration of sucrose during freezing			
		0.0	0.0029 M	0.011 M	0.044 M
Toxicity, LD_{50}	Untreated	130			
	Frozen and thawed	<7	<7	17	67
	Frozen, then incubated with coenzymes	17	55	170	170
Hemolytic activity, end-point	Untreated	180			
	Frozen and thawed	7	10	53	151
	Frozen, then incubated with coenzymes	77	128	157	220
Respiratory activity, *μl./ml./hr.*	Untreated, no coenzymes	80			
	Untreated, with coenzymes	134			
	Frozen, and thawed, no coenzymes	2	6	23	62
	Frozen and thawed, coenzymes added	53	65	93	120

The figures for LD_{50} represent the denominator of the dilution required to kill 50 per cent of the mice.

The figures for hemolytic end-point represent the denominator of the dilution required to give an optical density reading of 0.3 in the hemolysin assay.

In the oxygen uptake experiments each flask contained glutamate, 0.014 M; $MgCl_2$, 0.002 M; $MnCl_2$, 0.00033 M; potassium phosphate, 0.01 M; KCl, 0.12 M, and 1 ml. of rickettsial suspension. The rates given are those observed between the 2nd and 4th hours, as in several instances there was an increase in rate with the frozen preparations during the first 2 hours. DPN, 0.36 mg./ml.; CoA, 0.13 mg./ml.; and GSH, 0.0013 M were present in those flasks containing coenzymes.

In the toxicity and hemolysin experiments the rickettsial suspensions were assayed before freezing, "untreated." After freezing and thawing twice, each was diluted one to ten into the basal medium described in the text, and containing also DPN, CoA, and GSH. Each was assayed at once, then incubated at 34°C. for 3 hours and again assayed.

M sucrose was chosen as the freezing medium, as it produces a marked drop in activity on freezing, while still allowing extensive recovery. Preliminary experiments indicated that the reactivation reaction was fairly rapid. It had usually reached 75 to 80 per cent of its maximum value in 1 hour at 34°C. and showed no further change after 2 hours. It can be seen from Experiment 3 (Table II) that reactivation is much slower at 0° than at 34°C.

TABLE II

The Effect of DPN and CoA on the Restoration of Hemolytic Activity and Toxicity of Typhus Rickettsiae after Inactivation by Freezing

Conditions of incubation	Hemolytic activity		Toxicity	
	Before incubation	After incubation	Before incubation	After incubation
	End-point	End-point	LD_{50}	LD_{50}
No coenzymes	8	8	<7	<7
DPN and CoA	8	65	<7	55
No coenzymes	9	10		
DPN	10	25		
CoA	10	10		
DPN and CoA	10	37		
No coenzymes	16	15	7	<7
CoA	17	33	7	7
DPN	16	54	<7	17
DPN and CoA	16	126	7	30
DPN and CoA, at 0°C.	16	32	7	14

For the significance of the figures for the toxic and hemolytic end-points see footnote to Table I.

TABLE III

The Effect of DPN and CoA on the Oxidation of Glutamate by Typhus Rickettsiae after Inactivation by Freezing and Thawing

Preparation No.	Treatment	Coenzymes added		
		None	DPN	DPN, CoA, and GSH
		microliters O_2 per ml. per hr.		
5842	None	119	167	195
5842	Frozen in isotonic sucrose	90	156	166
5842	Frozen twice in 0.0029 M sucrose	0.6	40	102
5552	None	153		215
5552	Frozen twice in 0.0029 M sucrose	18	74	125

These experiments were carried out in the same way as those given in Table I. Coenzymes, when added were used in the concentrations given there.

The data given in Tables II and III show that addition of DPN is essential for restoration of hemolytic activity, toxicity, and respiration, and that CoA further increases this recovery, although it is without marked effect in the absence of DPN. The other constituents of the basal medium are without

effect on the reactivation with the possible exception of the heated normal yolk sac. With some preparations of rickettsiae this seems to be required for maximal reactivation, with others it is not. This is probably related to the amount of yolk protein still contaminating the rickettsiae, since the more completely the rickettsiae have been separated from yolk material, the more unstable they become in the absence of a small amount of added yolk sac protein.[1]

The restoration of hemolytic activity brought about by DPN and CoA is of the same order of magnitude in a basal medium made isotonic with KCl as in the sucrose medium used in the above experiments. The results obtained with these two coenzymes naturally suggest that other coenzymes might further increase the recovery of the activity lost on freezing. Experiments along this line are still in progress, but so far no other coenzyme has been found to yield consistent increases.

It seemed of interest to determine whether or not changes in infectivity of the rickettsiae analogous to those observed in toxicity and hemolytic activity were taking place. For these experiments serial tenfold dilutions of the rickettsial suspension were prepared (*a*) before treatment, (*b*) after freezing and thawing twice in the absence of sucrose, and (*c*) after incubation of the latter preparation for 3 hours in the presence of DPN and CoA. Two tenths ml. aliquots of the 10^{-3}, 10^{-5}, and 10^{-7} dilutions of the original suspension, and similar aliquots of the 10^{-3} and 10^{-5} dilutions of the treated rickettsiae were injected into the yolk sac of 7 day embryonated eggs, using 12 eggs per group. The eggs were incubated at 34°C. and were candled daily. The average day of death of eggs dying between the 3rd and the 14th day postinoculation was calculated for each group and is considered to be a measure of the number of infective rickettsiae inoculated. The data of one typical experiment are illustrated in Fig. 1. Curve 1 represents the death rate of the untreated rickettsiae. For curves II and III the suspension was frozen and thawed twice, then diluted 1 to 10 into three separate flasks containing basal medium and coenzymes. Each was titrated at once, giving the points indicated on curve II, then incubated for 3 hours at 34°C. and titrated again, giving the points on curve III. It is evident that there is a considerable drop in infectivity after freezing and that this loss is partially reversed by treatment with DPN and CoA. The changes observed in infectivity appear less marked than those recorded above in hemolytic activity and toxicity, due to the greater accuracy of the latter methods, which make possible the detection of smaller relative differences in activity. The data in this infectivity experiment actually indicate that there was about a 300-fold decrease in infectivity after freezing and that the infectivity of the frozen organisms increased approximately 15-fold after in-

[1] Bovarnick, M. R., unpublished results.

cubation with coenzymes. Other experiments have given similar results and have indicated that infectivity is restored only in the presence of coenzymes.

The requirement for DPN and CoA for restoration of all those properties of the rickettsiae that are lost after freezing suggested that the rickettsiae normally contain these coenzymes and that they are rendered rapidly diffusible by the

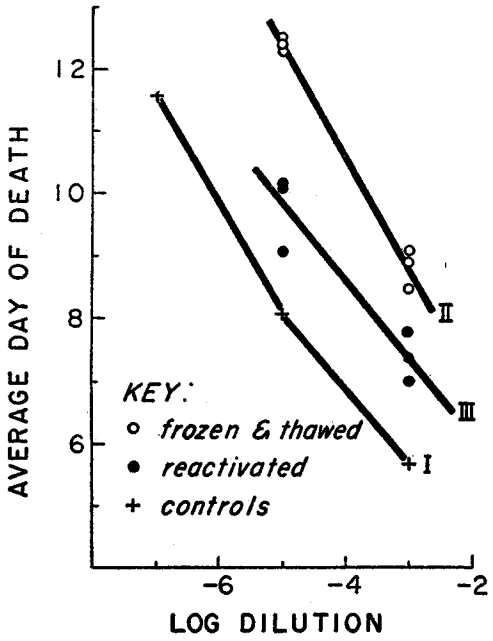

FIG. 1. Changes in the infectivity of rickettsiae for eggs after freezing and subsequent incubation with DPN and CoA. Plus signs, time of death of eggs inoculated with untreated rickettsiae. Open circles, time of death of eggs inoculated with rickettsiae frozen and thawed twice in isotonic salt solution. Filled circles, time of death of eggs inoculated with the same frozen rickettsiae after incubation for 3 hours with DPN and CoA.

process of freezing and thawing. In order to test this hypothesis in the case of DPN, the rickettsial suspensions were centrifuged for 45 minutes at 5000 R.P.M. The precipitated rickettsiae and the supernatant solutions were analyzed separately for DPN as described under Methods. One complete analysis of a suspension of rickettsiae that had been frozen and thawed in isotonic salt solution is given in Table IV. Each TCA extract was tested with and without an additional known amount of DPN, as indicated in column 3 to be sure that low apparent values of DPN were not due merely to inhibition by the

extract. The values given in column 2 represent the volume of the original fraction used in the assay. The DPN values given in column 6 were calculated on the assumption that the rate with any given DPN concentration in the TCA extracts is only 74 per cent of that observed in the controls, as discussed under Methods.

TABLE IV

Analysis of a Frozen and Thawed Preparation of Rickettsiae for Distribution of DPN

Fraction analyzed	Volume assayed	DPN added	Rate of CO_2 evolution	Rate of CO_2* evolution corrected	DPN‡ found	DPN§ concentration
	ml.	*μg.*	*μl./30 min.*	*μl./30 min.*	*μg.*	*μg./ml.*
Whole suspension	0.78	0	45.6	60.8	1.33	1.70
	0.78	0.43	54.2	72.2	1.65	1.56
Precipitate	0.91	0	4.6	6.0	0.04	0.04
	0.91	0.71	29.1	38.8	0.77	0.07
Supernatant	0.88	0	43.1	57.4	1.25	1.42
	0.88	0.29	53.4	71.2	1.62	1.51
	0.79	0	38.8	51.6	1.09	1.40
	0.79	0.43	47.6	63.4	1.41	1.26
—	—	0	4.6			
		0.14	10.7			
		0.29	16.6			
		0.71	37.7			
		1.42	64.2			

* Rate values corrected for the 25 per cent reduction in rate found in trichloracetic acid extracts of normal yolk sac particles.

‡ DPN values calculated from the corrected rate values by means of a curve constructed from the rates found with DPN alone (*cf.* lower half of table).

§ DPN concentration in the original fraction analyzed, corrected for DPN added during analysis (*cf.* column 3).

Table V summarizes the results of similar analyses of several untreated and frozen preparations. With untreated preparations DPN was invariably found in the precipitate, little or none in the supernatant. With rickettsiae that had been frozen and thawed in the absence of sucrose, no DPN was found in the precipitate, and variable results were obtained with the supernatants. In some cases (*cf.* preparations 5552 and 5701) most of the original DPN was detectable in the supernatant, in others (*cf.* preparation 5842), there was none. These discrepancies are probably due to the presence of varying amounts of a DPNase in the rickettsial preparations, probably in the contaminating yolk material. When DPN in a concentration approximately equivalent to that found in the

rickettsial preparations was added to a preparation of particles from normal yolk sac, the DPN had completely disappeared after 1 hour at 0°C. It has frequently been observed that different washed preparations of rickettsiae differ in their content of normal yolk antigen, as estimated by complement fixation tests with anti-yolk serum. This may thus account for the variations found in the behavior of the diffusible DPN in different pools.

TABLE V

The Effect of Freezing and Thawing on the Distribution of DPN in Suspensions of Typhus Rickettsiae

Preparation No.	Treatment	Fraction analyzed	DPN concentration
			μg./ml.
5552	None	Precipitate	1.31
		Supernatant	0.16
5552	Frozen and thawed twice in 0.0029 M sucrose	Whole suspension	1.64
		Precipitate	0.05
		Supernatant	1.30
5842	None	Whole suspension	1.09
		Precipitate	0.96
		Supernatant	0.0
5842	Frozen and thawed twice in 0.0029 M sucrose	Whole suspension	0.90
		Precipitate	0.13
		Supernatant	0.0
5701	Frozen and thawed twice in 0.0029 M sucrose	Whole suspension	0.93
		Precipitate	0.15
		Supernatant	0.67

DISCUSSION

It seems apparent from the above results that when rickettsiae are frozen and thawed in isotonic salt solution their DPN, which is normally held in a non-diffusible (or slowly diffusible) form, is rendered diffusible and rapidly lost. Presumably their CoA is similarly lost. It is not surprising that such coenzyme-deficient organisms should have lost their ability to oxidize glutamate and that this property should be restorable merely by addition of the lost coenzymes. Since there is already evidence that the hemolytic activity of the rickettsiae depends upon the presence of glutamate (7), it is perhaps understandable that this property also should depend upon the presence of the coenzymes that make possible the utilization of glutamate. It seems, however, quite unexpected that such properties as toxicity and infectivity should be

restorable merely by addition of these coenzymes. Especially in the case of infectivity one would expect either that it would be irreversibly destroyed, or that if the only damage done to the rickettsiae were that of removal of coenzymes, the infectivity would appear to be unchanged, since tissue cells should be able to supply these substances. The reversible changes actually observed suggest either that cellular coenzymes are unavailable, or that possibly the presence of coenzymes in the rickettsiae is a prerequisite to their ability to penetrate the cells.

It should be noted that the reactivation of the rickettsiae by DPN and CoA is not an instantaneous reaction. The titres obtained immediately after mixing rickettsiae and coenzymes are no different from those obtained in the absence of the coenzymes. Also the reaction proceeds very much more slowly at 0°C. than at 34°C.

It is of interest that two coenzymes, adenosinetriphosphate and coenzyme A, have also been found to be of importance for the *in vitro* survival and multiplication of another intracellular parasite, *Pl. lophurae* (8, 9).

There are several analogies in other biological systems to the reversible loss of activity by the rickettsiae. Possibly the closest similarity is in the changes brought about in mitochondria on freezing. It has recently been shown (10) that the loss in oxidative ability of rat liver mitochondria that occurs on freezing can be partially reversed by addition of DPN and ATP, and that the DPN of such frozen mitochondria has become non-sedimentable. If, however, the mitochondria, like the rickettsiae, are frozen in isotonic sucrose, a large part of their oxidative ability remains unimpaired even without added coenzymes (10, 11). Among the bacteria and viruses photoreactivation of organisms treated with ultraviolet light has been observed many times (12, 13). More recently a reversal of the apparent loss of viability of bacteria that had been treated with heat, alcohol, or chlorine has also been observed (14). However, the reversal in the latter case was brought about by incubation of the bacteria in the presence of certain metabolites, especially the dicarboxylic acids, and a much longer period of time, 24 hours, was required than is necessary either for photoreactivation of bacteria or for coenzyme reactivation of the rickettsiae, so that it is more difficult to exclude completely the possibility of growth of undamaged organisms. Recent results in this laboratory indicate that reversible losses in the activity of rickettsiae may also be brought about by methods other than freezing, although the results are not yet as reproducible as those obtained by freezing. Thus changes in microorganisms that result in loss of their ability to multiply, or to exhibit other characteristic activities, under standard assay conditions and that can be reversed by suitable treatment, may be of rather widespread occurrence and make difficult determination of the true number of viable organisms in any cell population.

SUMMARY

Rickettsiae that have been frozen and thawed in isotonic salt solutions show greatly decreased toxicity for mice, hemolytic activity, respiration, and infectivity for eggs. All these properties can be partially restored by incubation of the rickettsiae in the presence of DPN and coenzyme A for 2 hours at 34°C.

The extent of both inactivation and of subsequent reactivation is markedly affected by the presence of low concentrations of sucrose during the process of freezing and thawing.

It has been shown that DPN is present in rickettsial suspensions and that in preparations that have not been frozen, the DPN sediments with the rickettsiae. After freezing in isotonic salt solution the DPN becomes non-sedimentable.

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