FACTORS INFLUENCING THE SURVIVAL OF SPIROCHETES IN THE FROZEN STATE*

By THOMAS B. TURNER, M.D., and NANCY L. BRAYTON

(From the Department of Bacteriology of The Johns Hopkins School of Hygiene and Public Health, Baltimore)

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It has been shown previously that various types of spirochetes and filtrable viruses when stored at a temperature of −78°C. retain their virulence and pathogenicity for laboratory animals for periods up to 3 years. Storage at higher temperatures, −10°C. or −20°C., however, is followed, in the case of spirochetes, by loss of virulence and death of the organisms within a period of days or weeks (1, 2). The explanation of this phenomenon is not clear. A number of theories have been advanced by different investigators but none seems altogether satisfactory. In this paper will be presented a series of observations which serve to reveal some of the factors that influence survival or death of spirochetes during the freezing process.

The preservation of living organisms in the frozen state may be divided into three steps. First, the process of cooling from the unfrozen state to the lowest temperature employed; second, storage at a fixed temperature for long or short periods of time; third, the process of warming from the storage temperature to that of the unfrozen state. Conceivably, during each of these periods events may transpire which materially affect the survival or virulence of the organism in question. In the experiments reported below the technique employed in one or another of these steps has been altered, and the effect on the test organism observed.

Experimental Method

The studies were made on the spirochete of relapsing fever (genus, Borellia Swellengrebel) because experiments with this organism are technically less difficult to perform than experiments with any of the other readily available spirochetes. Adequate quantities of blood or serum rich in relapsing fever spirochetes can be obtained from rats within 2 to 5 days after inoculation; the organism can be easily visualized, microscopically;

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and, of particular importance in these studies, the infectivity of a given specimen can be determined within a short time by the inoculation of mice. Within from 24 hours to several days after intraperitoneal inoculation, depending largely on the dosage of spirochetes, white mice develop a blood stream infection and motile spirochetes can be readily demonstrated by dark field microscopic examination of a drop of blood from the tail vein. Spirochetes are demonstrable in the blood stream for 3 to 5 days. The first attack may be followed by one or more relapses; mice rarely die of the disease.

The *novyi* strain of relapsing fever spirochetes was mainly used, although occasionally, as noted below, the *duttoni* and Tickmouse II strains were employed. Rats showing a heavy blood stream invasion were bled from the heart and the pooled blood defibrinated. Commonly, the blood was centrifuged and only the plasma used for freezing, but occasionally whole defibrinated blood was employed. However, the material used in any given experiment was always the same, and tests were made promptly after removal of blood from the rats.

Of the available indices to survival or death of relapsing fever organisms preliminary experiments indicated that pathogenicity for mice is the most reliable, and this index has been used in determining the results of these experiments. Study of the motility and morphology of the spirochete provides a rough index to survival, but these methods are regarded as unreliable and not sufficiently accurate to bring out anything but pronounced differences. Aside from inherent biologic factors, motility is influenced by environmental factors such as temperature of the medium, length of time outside the animal body, etc. Moreover, technical factors incident to making motility counts lead to some variation. Morphology of the organism, likewise, was found not to be an accurate guide to pathogenicity. Specimens which contained a high proportion of distorted forms were as a rule not pathogenic for mice in as high dilution as were specimens in which most of the spirochetes appeared to be normal, but the correlation was not close.

Virulence tests in mice were made by injecting spirochete-containing material, properly diluted with infusion broth, intraperitoneally in 0.5 cc. amounts. 3 mice were commonly inoculated with each dilution. Blood from the tail was examined daily for the presence of spirochetes, and the day following inoculation on which the first of the 3 mice became positive was regarded as the incubation period. Ordinarily each of the 3 mice became positive within 24 hours of each other, although in titrations some irregularity in this respect was noted as the end point was approached. In most instances, however, differences of 2 days or more in the incubation period may be considered significant. On the basis of a large number of observations it was evident that there was a fairly close correlation between the incubation period in mice and the end point of the titration. Consequently in many of the experiments the incubation period of a specific dilution has been used as an index to the pathogenicity of the material tested.

EXPERIMENTAL RESULTS

Effect of Freezing on Morphology and Motility.—When relapsing fever spirochetes are frozen at −78°C. and thawed under optimum conditions an increase in the proportion of distorted forms can sometimes be noted. Deviation from normal morphology usually consists of shrinkage of the body of the spirochete, angulation in place of the smooth regular curves, and at

times granular-like changes in the cell protoplasm. When frozen and thawed under adverse circumstances, distorted forms are more constantly observed.

After freezing and thawing, even under optimum conditions, great variation in the proportion of motile organisms was observed. In some specimens there was apparently quite as high a proportion of motile forms as was observed in the fresh material, while in other specimens a marked decrease in motility was noted. No close correlation between motility and virulence for mice was found in a large series of tests. Moreover, the proportion of motile to non-motile organisms varied markedly at different periods after thawing.

Immediately after thawing, most of the organisms are inactive. As the medium approaches room temperature more organisms become motile, but in many instances the maximum percentage of motile spirochetes is not observed until 1 to 2 hours after thawing. Motility counts (500 organisms for each count) were made on 7 specimens at intervals from 10 minutes to 3 hours after thawing, the specimens being at room temperature during this period. Maximum motility was noted after 2 hours in the majority of specimens but in some the maximum was reached in 1 hour. In one sample, for example, at 10 minutes no motile organisms were seen; at 30 minutes 20 per cent of the spirochetes were motile; at 1 hour 70 per cent; and at 2 hours and 3 hours, respectively, 90 per cent. Even allowing for possible technical error the differences are quite pronounced. In these tests it was definitely determined that the serum in which the organisms were suspended had reached room temperature (approximately 22°C.) within 10 minutes after thawing in the water bath.

Evidently, the return of motility of spirochetes after thawing is influenced by factors other than the temperature of the medium in which they are suspended. It has been amply demonstrated that material containing few or no motile forms may be highly infective for mice.

Effect of Freezing on Infectivity.—Five different specimens of rat blood plasma containing numerous spirochetes per microscopic field were tested both before and after freezing. Specimens were frozen and maintained at −78°C. Thawing was carried out at 37°C., either in the water bath or in the incubator. The results of this experiment are given in Table I.

In each instance specimens showed evidence of a decrease in titre after freezing as compared with that before freezing. In the specimens on which titrations were made, there was a fall in titre from 10^{-8} to 10^{-6} in 2 specimens, and from 10^{-7} to 10^{-5} in the remaining one. In all, a prolongation of the incubation period was noted. These changes were not correlated with the duration of storage at −78°C., for they were observed after 5 days as well as after 6 months. As will be noted later, probably the greatest damage occurred during the thawing period.

Results similar to the above were obtained by Winchester and Murray (3) in the study of the effect of freezing on bacteria. Standardized suspensions of *Bacillus typhi* were frozen and stored at the temperature of liquid air (−192°C.) and one sample was tested daily for 10 days. After 1 day there was a drop from 1,000,000 viable organisms per cc. in the fresh suspensions to 10,000 per cc. in the frozen sample, but samples tested on succeeding days showed no further drop. Tests on samples of the same material after storage for 13, 16, and 19 months gave variable results, but there was no consistent decrease with the lapse of time. As in the case of relapsing fever spirochetes, the principal injury at this low temperature

TABLE I

Comparison of the Virulence of Relapsing Fever Spirochetes before and after Freezing at −78°C.

Specimen No.	Period of storage at −78°C.	Results				
		Incubation period, days			Titration dilution end point	
		Dilution tested	Before freezing	After freezing	Before freezing	After freezing
8	4 days	10^{-1}	2	3	—	—
6	5 "	10^{-5}	3	4	10^{-7}	10^{-5}
4	6 wks.	10^{-6}	4	9	10^{-8}	10^{-6}
N	6 mos.	10^{-5}	2	5	10^{-8}	10^{-6}
D	10 "	10^{-3}	2	8	—	—

apparently occurred either during the cooling or the warming process, and not during the storage period.

These observations are particularly interesting in view of the results of titrations made with the viruses of influenza, yellow fever, and spontaneous encephalomyelitis before and after freezing (1). In the case of these viruses there was little or no evidence indicative of a decrease in titre with freezing. Since the spirochete of relapsing fever, and even the typhoid bacillus, are considered to be more complex structures than are filtrable viruses, perhaps this factor accounts for the latter's being less susceptible to damage by freezing at low temperatures.

Effect of Repeated Freezing and Thawing.—Material containing relapsing fever spirochetes retained its infectivity for mice after having been frozen and thawed under optimum conditions as many as 3 times, but not after 5, 7, and 9 times. The results seemed to have been unaffected by the duration of storage. As would be expected from the results of experiments cited in the preceding paragraph, the titre apparently decreases with each

refreezing and the number of refreezings required to destroy all spirochetes will depend in part on the number of organisms originally present. Some microorganisms, however, survive repeated refreezing. Kyes and Potter (4) have shown that cultures of avian tubercle bacilli subjected 200 times to alternate freezing in liquid nitrogen (−195.5°C.) and thawing in water at 34–36°C. still showed some viable organisms.

Effect of Freezing and Maintenance Temperatures Other than −78°C.—In a previous paper (1) it was shown that *Treponema pallidum* frozen rapidly at −78°C. and after 2 hours transferred to a temperature of −20°C. did not survive as long as 2 months. Samples of the same material frozen at −20°C. and maintained at −78°C. for 2 months were highly pathogenic for rabbits. This organism was also adversely affected by storage at −10°C. for several days. Those experiments have been repeated and extended using relapsing fever spirochetes as the test organism.

The test material consisted of 3 large lots (specimens 1, 2, and 4) of pooled rats' plasma rich in spirochetes, although the number of organisms per microscopic field varied with each lot. Various samples of each large specimen were treated in different ways as indicated in Table II. Some were frozen and maintained at −78°C., others were frozen at −78°C. and transferred to an electrically operated refrigerator at −20°C. or to an ice-salt mixture in an insulated container at −12°C. Other samples were frozen at −10°C., −12°C., or −20°C. and maintained at −12°C. or −20°C., or transferred, after varying periods of time, to −78°C. After periods of 1 day to 6 weeks samples were thawed, the motility of the spirochetes determined by dark field microscopic examination, and the pathogenicity of the material tested in mice. The results of these experiments are shown in Table II.

Direct comparison can be made only among samples of the same specimen, although in general the results are consistent among all specimens.

Tests made on samples of specimen 1 show that maintenance at −12°C. for even one day causes a pronounced decrease in pathogenicity, regardless of whether the material was frozen at −78°C. or at −12°C. The experiments on specimen 2 show that there was little difference between the results with samples frozen and maintained at −78°C., and those frozen at −20°C. for 2 hours and maintained at −78°C. for 1 to 4½ weeks. On the other hand, samples frozen at −20°C. or at −78°C. and maintained at −20°C. as a rule showed a decrease in pathogenicity. The tests made on specimen 4 confirm the preceding results in that maintenance for 6 weeks at −20°C. caused a marked reduction in virulence as compared with the sample maintained at −78°C.

When freezing is carried out at −20°C. or at −10°C., and this period is prolonged to 1, 2, or 3 days, the results vary with the duration of the freezing period and with the temperature to which the organisms were subjected during this period. Freezing at −10°C. was more detrimental than at

−20°C., and at either temperature prolongation of the period to 3 days caused more injury to spirochetes than did exposure for only 1 day. It is clear that periods of 1 day or longer should be considered as maintenance periods, rather than as the period of freezing.

TABLE II

Effect of Freezing and Storage Temperatures Higher than −78°C. on the Virulence of Relapsing Fever Spirochetes

Specimen No.	Freezing period		Storage period		Results			
	Temperature	Duration	Temperature	Duration	Motility 2 hrs. after thawing	Incubation period: Dilution tested	Incubation period: Days	Titration end point
	°C.		°C.					
1	−78	—	−78	4 days	+	10^{-1}	2	
	−78	½ hr.	−12	1 day	0	"	7	
	−12	—	−12	" "	0	"	7	
2	−78	—	−78	1 wk.	++++	10^{-2}	1	
	−20	2 hrs.	−78	" "	++++	"	1	
	−78	" "	−20	" "	+	"	2	
	−20	—	−20	" "	+++	"	1	
	−78	—	−78	3 wks.	++++	10^{-2}	1	
	−20	2 hrs.	−78	" "	++++	"	1	
	−78	" "	−20	" "	+	"	3	
	−20	—	−20	" "	+++	"	2	
	−78	—	−78	4½ wks.	++++	10^{-3}	3	
	−20	2 hrs.	−78	" "	++++	"	3	
	−78	" "	−20	" "	+	"	6	
	−20	—	−20	" "	+++	"	5	
4	−78	—	−78	6 wks.	+++	10^{-2}	1	10^{-6}
	−78	2 hrs.	−20	" "	+	"	Neg.	10^{-1}
	−20	1 day	−78	" "	++	"	2	10^{-6}
	−20	2 days	−78	" "	+	"	3	10^{-5}
	−20	3 "	−78	" "	+	"	3	10^{-5}
	−10	1 day	−78	" "	++	"	5	10^{-4}
	−10	2 days	−78	" "	+	"	5	10^{-4}
	−10	3 "	−78	" "	+	"	6	10^{-3}

The studies of Haines (5) are of interest in this connection. This investigator, working with various types of bacteria, found considerable variation in survival among species subjected to rapid freezing at −70°C. and thawing at room temperature. Tests with one species, *Bacillus pyocyaneus*,

however, showed no appreciable difference in survival when frozen at −5°, −20°, or −70°C. provided they were thawed soon afterwards. Haines also noted that the proportion of organisms surviving showed close correlation with the storage temperature. Organisms died out slowly at −20°C., a fair proportion being still viable after approximately 6 months, but at higher temperatures, −10°, −5°, −3°, −2°, or −1°C., death occurred much more rapidly.

These experiments with spirochetes and certain bacteria show very clearly that temperatures of −20°C. or higher are not favorable to the survival of these organisms over long periods. The rate at which organisms die during the storage period is probably correlated with temperature, but it is also probable that the rate varies according to the species of pathogen. All the evidence indicates that spirochetes suffer greater damage at −20°C. than do many bacteria, and some incomplete data of the authors suggest that influenza virus (PR8 strain) undergoes less loss of virulence during the same period of time at −20°C. than do either these bacteria or spirochetes. The available data are yet too few, however, to permit of broad generalization, for it is quite possible that considerable variation in this respect occurs within the larger groups of bacteria or filtrable viruses.

Effect of Various Rates of Freezing and Thawing.—The results of the foregoing experiments suggest that the rate at which relapsing fever spirochetes are cooled from the unfrozen state to −78°C. may not be an important factor in their survival. In contrast to this, however, it was found that the rate at which the temperature is raised from −78°C. to above 0°C. is exceedingly important. Rapid thawing is far more conducive to the survival of spirochetes than is slow thawing. The following experiments were designed to provide more accurate information on these points.

When vials containing amounts of material such as were used in these experiments (1 to 5 cc.) are placed in a mixture of solid carbon dioxide and 95 per cent alcohol, solidification occurs almost instantaneously and the material reaches −65°C. to −70°C. within about 30 seconds. The rate of temperature reduction, however, is not uniform. This will be termed immediate freezing. Slower and more uniform rates of freezing may be attained by the addition at regular intervals of small pieces of solid carbon dioxide to alcohol, not previously chilled, in an insulated container. The effect on the survival of relapsing fever spirochetes of slow uniform rates of freezing as compared with immediate freezing is shown in Table III. In general slow freezing over periods up to 10 hours seemed to exert little or no adverse effect on the viability or pathogenicity of these spirochetes.

In all except the tests on specimen 1, no significant difference was noted in the motility of spirochetes or in the virulence for mice of material frozen slowly as compared with samples of the same specimen frozen rapidly.

Under ordinary laboratory conditions frozen specimens were brought back to the unfrozen state by warming in the water bath at 37°C. or warming to room temperature

TABLE III

*Effect of Slow Freezing on the Virulence of Relapsing Fever Spirochetes**

Specimen No.	Freezing time	Duration of storage at −78°C.	Results			
			Motility 2 hrs. after thawing	Incubation period		Titration end point
				Dilution	Days	
1	Immediate	4 days	++	10^{-1}	2	
	10 hrs.	" "	0	"	7	
2	Immediate	4½ wks.	++++	10^{-3}	3	
	2 hrs.	" "	++++	"	3	
	10 "	" "	++++	"	3	
6	Immediate	5 days	++++	10^{-4}	4	10^{-5}
	½ hr.	" "	+++	"	4	"
	2½ hrs.	" "	++++	"	4	"
8	Immediate	3 wks.	+	10^{-1}	3	
	6 hrs.	" "	+	"	3	
9	Immediate	5 days	++	10^{-3}	3	
	2 hrs.	" "	++	"	3	
	6 "	" "	++	"	4	

* Samples thawed at 37°C. or at room temperature, but method of thawing consistent for samples of each specimen.

at approximately 22°C. In the water bath, thawing occurred in about 30 seconds' while at room temperature thawing was not complete before 20 to 35 minutes had elapsed, depending somewhat on the volume of material. In either case, however, the rate of thawing was not uniform throughout, since the rate depends upon the difference in temperature between the surrounding medium and the container, and as this difference decreases the rate of warming decreases. For experimental purposes other irregular rates of thawing were obtained by placing specimens in the incubator at 37°C., in which case thawing was not complete before about 25 minutes had elapsed, and by using insulated containers, in which case the total period required for thawing could be regulated, within limits.

Uniform rates of thawing were obtained by electrically warming an alcohol medium

from −78°C. to 0°C.[1] Alcohol was placed in an insulated container (Dewar flask). The heater was operated by a 110 D.C. current. Into the circuit were introduced two resistance coils and a voltmeter. To insure uniform distribution of heat, a stirrer, propelled by an outside air driven turbine, was placed in the flask. In such a system

TABLE IV

*Effect of Slow Thawing on the Virulence of Relapsing Fever Spirochetes Frozen and Stored at −78°C.**

Specimen No.	Rates of thawing		Results		
	Method	Time	Motility 2 hrs. after thawing	Incubation period	
				Dilution tested	Days
3	Water bath at 37°C.	30 sec.	++++	10^{-4}	2
	Incubator at 37°C.	25 min.	+++	"	3
	Room temperature	35 "	+++	"	3
	Insulated container	2½ hrs.	0	Whole blood	Neg.
	" "	7 "	0	" "	"
6	Water bath at 37°C.	30 sec.	++++	10^{-4}	4
	Room temperature	30 min.	+++	"	5
	Insulated container	2½ hrs.	0	"	Neg.
8	Water bath at 37°C.	30 sec.	+	10^{-1}	3
	Room temperature	6 min.†	+	"	4
	Ice box 4°C.	12 " †	+	"	3
	Uniform	10 min.	+	"	3
	"	" "	+	"	3
	"	30 "	+	"	5
	"	" "	+	"	4
	"	6 hrs.	0	"	Neg.
	"	" "	0	"	"
9	Water bath at 37°C.	30 sec.	++	10^{-2}	3
	Insulated container	2 hrs.	0	Whole blood	Neg.
	" "	6 "	0	" "	"

* All specimens frozen rapidly at −78°C. Duration of storage varied, but was the same for all samples of each specimen.

† Relatively quick thawing due to small volume of material.

the rise in temperature per minute is proportional to the square of the voltage, and the desired rate of warming could be calculated. A range of warming rates from 0.1°C. to 8.5°C. rise per minute could be obtained.

[1] This apparatus was kindly designed and constructed for us by Dr. Robert Krieble, who was then a postgraduate student in the Department of Chemistry of The Johns Hopkins University.

The effect on relapsing fever spirochetes of various rates of thawing is shown in Table IV. It will be noted that there is a slight but consistent prolongation of the incubation period of samples thawed at room temperature (approximately 22°C.) as compared with water bath temperature (37°C.), while in the case of samples thawed over a period of 2 to 7 hours all spirochetes were apparently killed, since no motility was observed and the material was not infectious for mice in the dilutions tested. The results obtained with uniform rates of thawing were essentially the same as those obtained with irregular rates.

It is evident from these experiments that slow warming from −78°C. to 0°C. is much more harmful to spirochetes than is slow cooling from 0°C. to −78°C. Why this should be true is not clear. It has been suggested that the temperature change incident to either freezing or thawing is the important factor and, since spirochetes which are being warmed have always been first subjected to cooling, the cooling episode renders them more susceptible to the temperature changes incident to thawing. This hypothesis was shown not to be valid by the following experiment:

Three samples of one specimen (No. 8) were frozen rapidly and thawed rapidly. One sample was then frozen rapidly again, as a control, and the other 2 samples were frozen slowly over a period of 6 hours. All samples were then thawed rapidly and the 10^{-1} dilution tested for virulence. The incubation period of the control sample was 5 days, and of the other 2 samples 6 and 8 days, respectively. Although the samples cooled slowly showed evidence of slight damage, the damage was not nearly so great as regularly occurs when spirochetes are thawed slowly.

DISCUSSION

The practicability of maintaining various types of pathogens at the temperature of solid carbon dioxide has been amply demonstrated. Infectious agents belonging to the group of spirochetes, bacteria, and filtrable viruses have been preserved for periods up to 3 years (2). Under certain conditions of freezing, however, these organisms are adversely affected, and at some freezing temperatures death of the organisms occurs. Little direct evidence is yet available regarding the mechanism which permits survival of pathogens at one temperature and not at another. The experiments reported in this paper and the investigations of Haines (5), however, suggest that more than one factor is responsible for the injury that may occur during the freezing process.

One set of injurious factors are associated with the period of temperature change from 0°C. to −78°C., and from −78°C. to 0°C. or above. The warming process is potentially more damaging than the cooling process,

for prolongation of the former step from a few minutes to several hours leads to a pronounced decrease in the infectivity of the material. Even under optimum conditions, however, some organisms are damaged by this temperature change; the harmful effect is not great in the case of relapsing fever spirochetes, scarcely demonstrable at all in the case of the filtrable viruses tested, and variable in the case of certain bacteria (Haines). Under these circumstances the injury occurs quickly and without relationship to the degree or duration of the storage temperature.

On the other hand, damage to microorganisms by freezing may occur under very different circumstances. At a storage temperature of −78°C. no demonstrable loss of virulence occurs over a period of months or even years, but at higher temperatures, −20°C., −10°C., or warmer, spirochetes and bacteria suffer material damage. This injury takes place, not immediately but over a period of days or weeks. The rate and degree of damage seem to depend upon the storage temperature; injury occurs more rapidly at −10°C. than at −20°C. For many organisms damage begins to occur at temperatures lying somewhere between −78°C. and −20°C., but the effect of temperatures within this range has not been adequately studied, largely because of technical difficulties. No good comparative study has been made of the survival of organisms at temperatures colder than −78°C. Numerous workers have shown that a wide variety of bacteria, spirochetes, and filtrable viruses survive temperatures ranging from that of liquid oxygen, −183°C., to that of liquid helium, −269°C. (6), and the indications are that these temperatures are just as favorable to survival as a temperature of −78°C.

At the risk of oversimplification, then, it can be said that in the maintenance of microorganisms at low temperatures the injury may arise from two sets of factors. The one, associated with the act of freezing and thawing, and the other, associated with the storage period. It seems probable that under the first set of conditions the damage is done by physical changes in the cell or in the surrounding medium. Various theories have been advanced attempting to explain the nature of this phenomenon; that the crystals formed at very low temperatures are smaller and hence less injurious to cells than the larger crystals formed at higher freezing temperatures; that in the slower freezing at relatively high temperatures water is extracted from the cell leaving a high concentration of salts which damages the cell. Neither of these theories seem adequately to explain why slow thawing is so injurious while slow cooling has relatively little harmful effect.

The damage that occurs during the storage period is often manifested gradually. At certain temperatures, for example, more organisms are

found dead after 6 weeks than after 2 or 4 weeks, and within the ranges tested the rate of death seems to vary according to the temperature. This suggests that at the higher temperatures injury is brought about mainly by changes incident to cell metabolism or to proteolytic or other enzymes, while at −78°C. these biological activities are suspended. Of interest in this connection is the observation of Smart (7) that cultures of certain bacteria, yeasts, and molds stored at approximately −9°C., showed slight growth over a period of 1 year, indicating that some biologic activity (division and growth) is possible, for those microorganisms at least, at that temperature.

SUMMARY

Titration experiments made with relapsing fever spirochetes before and after freezing showed the following:

1. With each freezing and thawing there is a slight but regular decrease in virulence, which decrease bears no relation to the duration of storage at −78°C. Ordinarily infectivity is destroyed by more than 4 refreezings.

2. There was not always close correlation between motility and infectivity.

3. Cooling spirochetes from 0°C. to −78°C. over a 2 to 6 hour period damages them only slightly more than does rapid cooling, but warming from −78°C. to 0°C. over a 2 to 6 hour period kills most of the organisms. Rapid thawing, as in a water bath, damages the spirochetes less than thawing more slowly, as at room temperature.

4. At storage temperatures of −12°C. and −20°C. there is a gradual decrease in virulence over a period of days or weeks, and by the 6th week the infectivity of the material is markedly reduced.

CONCLUSIONS

Optimum conditions for the preservation of spirochetes, and probably other microorganisms, in the frozen state are afforded by rapid cooling, storage at −78°C., and rapid thawing. These organisms are severely damaged by storage at temperatures of −20°C. or higher, and by slow thawing.

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