PROLONGED MAINTENANCE OF SPIROCHETES AND FILTRABLE VIRUSES IN THE FROZEN STATE*

By THOMAS B. TURNER, M.D., and WILLIAM L. FLEMING, M.D.

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

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In a previous paper (1) one of us described a practical method for preserving *in vitro* the pathogenicity of certain spirochetes and filtrable viruses by maintaining these organisms in the frozen state at a temperature approximating −78°C. It was shown that the virulence of the spirochetes of syphilis and of yaws was not appreciably altered after having been maintained at this temperature for one year, and that the viruses of human influenza (PR8 strain), yellow fever, and spontaneous encephalomyelitis of mice were infective for mice in virtually as high dilutions after having been frozen at −78°C. for 6 months as they were before freezing. It has now been over 3 years since these studies were begun, and we wish to record the results of virulence tests made on several types of spirochetes and filtrable viruses which have been maintained in the frozen state during this period.

The technique employed for freezing and maintaining infective material at −78°C. was briefly as follows:

To a 12 gallon insulated container partly filled with 95 per cent alcohol, solid carbon dioxide was added about twice weekly in order to maintain a temperature of approximately −78°C. Material to be frozen was placed in stoppered glass vials and immersed in the alcohol. Specimens were thawed at room temperature or, preferably, in the water bath at 37°C. This technique has been described more fully in a previous paper (1).

Spirochetes

Treponema pallidum and Treponema pertenue.—Soon after these studies were begun in the latter part of 1935, a number of specimens containing virulent *T. pallidum* and *T. pertenue* were frozen and stored at −78°C., as described above. These organisms were obtained from the testes of infected rabbits and were commonly frozen in a 20 per cent tissue sus-

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pension in infusion broth. After approximately 3 years these specimens were removed from the freezing mixture and thawed in the water bath at 37°C.; and the tissue suspension was inoculated into the testes of normal rabbits. The results of these tests are shown in Table I.

Four strains of *T. pallidum* were tested. The Nichols strain was isolated from a human case of syphilis in 1912 (2) and had been propagated in rabbits continuously until the time of these experiments. The other 3 strains, S4, S6, and S10, were obtained by

TABLE I

Results of Virulence Tests on Strains of Treponema pallidum and Treponema pertenue Stored at −78°C. for Approximately Three Years

Organism tested	Strain	Source Rabbit No.	Date spirochetes frozen	Date tested	Motile treponemes	Rabbit inoculated No.	Result in inoculated rabbit	Incubation period
			1935	*1938*				*days*
T. pallidum	Nichols	2-46	Dec. 9	Dec. 13	Many	4-81	Pos.	27
			1936	*1939*				
	"	2-89	Jan. 22	Feb. 6	"	5-51	"	20
	S4	2-79	" 8	Jan. 12	"	5-19	"	32
	S6	2-81	" 8	" 12	"	5-20	"	19
	S10	1-89	Feb. 20	Feb. 6	"	5-39	"	27
			1935	*1939*				
T. pertenue	YA	2-47	Dec. 19	Jan. 12	"	5-21	"	28
			1936					
	"	2-59	Jan. 22	Feb. 9	"	5-53	"	26
	YC	2-63	" 2	Jan. 12	"	5-22	"	24
	YD	2-56	" 10	Feb. 6	"	5-40	"	27
	YH	2-62	" 29	" 6	"	5-41	"	42
	YK	2-25	" 2	Jan. 12	None	5-23	"	33
	"	"	" 2	Feb. 6	Few	5-42	"	29

the senior author in 1934 from syphilitic patients living in Jamaica, B. W. I., and had been through 5 to 7 animal passages at the time of freezing (3). Upon thawing, all specimens showed many actively motile treponemes. Inoculation into the testis of a normal rabbit gave rise, after an incubation period varying from 19 to 32 days, to a typical syphilitic orchitis from which actively motile *T. pallidum* were obtained.

Although the virulence of only one of these specimens (No. 2-46) was tested before freezing, the results obtained with the frozen specimens indicate that no marked reduction in virulence occurred during the storage period. The incubation period of the lesions produced by the frozen material was, in each instance, within the range commonly observed with

fresh material. In character and extent the syphilitic orchitis was entirely similar to that produced by unfrozen syphilis spirochetes.

Similar tests were made on 5 strains of *T. pertenue*, all of which were obtained from patients in Jamaica, B.W.I., during the years 1932 to 1934 (3).

Strain YA had been through 18 to 19 animal passages at the time of freezing, and the others through 4 to 7 passages. All specimens, except those of strain YK, showed many motile treponemes after thawing, and upon intratesticular inoculations in normal rabbits gave rise to typical yaws lesions, within the usual incubation period observed when unfrozen material is employed. Although after thawing no motile spirochetes were noted in one specimen of strain YK, and only a few motile organisms in the other specimen, this material produced characteristic testicular lesions of yaws within the usual incubation period.

While none of the specimens was tested for virulence before freezing, the results of these tests indicate that no pronounced alteration in virulence had occurred during the 3 year period of freezing.

In addition to the specimens described above, virulence tests were made on 3 other specimens of syphilis spirochetes and 4 other specimens of yaws spirochetes, all of which had been stored at −78°C. for approximately 2 years. The results were essentially the same as in the tests previously mentioned. Innumerable specimens, comprising at least 20 different strains of syphilis and 8 of yaws treponemes, have been tested after storage for shorter periods, with similar results. It is quite evident that no appreciable alteration in virulence or pathogenicity occurs.

In order further to test the effect of prolonged storage at −78°C. on *T. pallidum*, samples of the same lot of infective material were tested before freezing and after various periods of storage.

A tissue suspension containing numerous *T. pallidum* was prepared from the testes of rabbit 2-46. One normal rabbit was inoculated intratesticularly with 0.3 cc. of this suspension before freezing. The remainder was distributed into vials, frozen and stored at −78°C. At 14 days, 1, 2, 4, and 6 months, and 1, 2, and 3 years after freezing, one sample of this lot was thawed and 0.3 cc. of the suspension inoculated intratesticularly in one or two normal rabbits. The results of these tests are shown in Table II.

All specimens showed numerous actively motile treponemes and each produced a typical syphilitic orchitis. In each instance the incubation period of the orchitis was within the range commonly observed with fresh material although, on the whole, it was somewhat longer for the frozen samples than for the one fresh sample of this lot that was tested. While this difference may be significant, it could also have been due to chance

alone. It should be noted, however, that there was no tendency toward prolongation of the incubation period with the lapse of storage time.

Relapsing Fever Spirochetes (Genus, Borrelia Swellengrebel).—It was shown by Jahnel (4) that relapsing fever spirochetes survive freezing at a temperature of −196°C., and Dr. Linda B. Lange using our storage facilities observed that these organisms were pathogenic for mice after storage at −78°C. for 11 months. Since then the authors, working with strains supplied by Dr. Lange, have amply confirmed those observations. Numerous specimens of the *novyi*, *duttoni*, and Tickmouse II strains have been tested after storage periods of a few weeks up to one year, almost invariably with positive results. After one year a specimen of strain Tickmouse II

TABLE II

Incubation Period of Syphilitic Lesions in Normal Rabbits Inoculated with the Same Lot of Infective Material, Which Had Been Maintained at −78°C. for Different Periods of Time

Period of storage at −78°C.	Incubation period in inoculated rabbit	Period of storage at −78°C.	Incubation period in inoculated rabbit
	days		*days*
Before freezing	16	6 mos.	23
14 days	32	6 "	24
1 mo.	22	1 yr.	22
2 mos.	22	1 "	33
4 "	18	2 yrs.	23
		3 "	27

Source of spirochetes, rabbit 2-46. Individual dose 0.3 cc.

spirochetes, upon intraperitoneal inoculation of rats, produced a heavy blood stream infection within 2 days after inoculation. Since in a subsequent paper (5) there will be presented a number of experiments made with relapsing fever spirochetes, no further details will be given at this time. Suffice it to say that these organisms regularly survive for long periods at −78°C.

Leptospira icterohemorrhagiae.—One strain of the spirochete of infectious jaundice, isolated from rats and propagated in guinea pigs, was tested after storage at −78°C. for 5 months and 10 months, respectively. Upon thawing, the specimens contained actively motile leptospira, but guinea pigs inoculated with this material died of infection caused by a bacterial contaminant present in the frozen material. Another strain of *L. icterohemorrhagiae,* which was of low virulence for guinea pigs and had been maintained on artificial media, was examined after having been frozen for

6 months. A large proportion of the organisms showed active motility. While no data are available, therefore, on the effect of freezing on the virulence of *L. icterohemorrhagiae* it is evident that these organisms remain viable at this temperature, as indicated by the presence of active motility.

The Spirochete of Rat Bite Fever.—A strain of *Spirillum minus* was recovered from a mouse which had been inoculated 7 days previously with rat blood. A number of passages in mice showed the period from intraperitoneal inoculation to invasion of the blood stream to be 6 to 8 days. After the 4th passage in mice, specimens of blood and peritoneal fluid containing actively motile *S. minus* were frozen and stored at −78°C. 6 months later a specimen of peritoneal fluid was thawed and inoculated intraperitoneally into mice. The inoculum contained motile spirochetes; and the blood of the inoculated mice, which was examined daily, became positive on the 18th day after inoculation. After one year a specimen of blood which contained numerous actively motile spirochetes was tested in the same manner. Each of 3 inoculated mice showed numerous spirochetes in the blood on the 11th day after inoculation. While the incubation period observed in these tests was somewhat prolonged, it is evident that *S. minus* survives at −78°C.

Filtrable Viruses

Virus of Human Influenza.—Experiments were made with the PR8 strain of influenza virus originally isolated by Francis (6). In August, 1936, mice which had been inoculated intranasally with the virus were killed, and a 10 per cent suspension in infusion broth was prepared from lungs showing typical consolidation. Serial dilutions were prepared from one portion of the fresh material and used for immediate inoculation. Other portions were frozen and stored at −78°C.

The virulence of the fresh material was tested by inoculating dilutions 10^{-4} to 10^{-8} intranasally in each of 6 white mice. Each mouse was given 0.03 cc. of the appropriate dilution. All mice inoculated with 10^{-4} dilution died between the 6th and 8th day and the lungs of each showed a type of consolidation characteristically produced by this virus. The remaining mice were killed on the 10th day after inoculation and their lungs examined. The lungs of all 6 mice inoculated with a 10^{-5} dilution showed extensive consolidation, while of the mice inoculated with a 10^{-6} dilution, the lungs of 5 showed a slight or moderate degree of consolidation and the lungs of one were normal. The lungs of mice inoculated with 10^{-7} and 10^{-8} dilutions were essentially normal.

Approximately 3 years later (June, 1939) a sample of this same lot of material, which had been stored at −78°C., was thawed in the water bath and serial dilutions prepared as in the case of the fresh sample. Each dilution from 10^{-1} to 10^{-8} was inoculated

intranasally in each of 4 white mice, 0.03 cc. being the individual dose. All mice inoculated with dilutions from 10^{-1} to 10^{-4}, inclusive, died between the 4th and 8th day, and the lungs of each showed typical consolidation. One of the mice inoculated with the 10^{-5} dilution died of pneumonia, and the remaining ones, killed on the 10th day, showed partial consolidation of the lungs. The lungs of mice inoculated with 10^{-6}, 10^{-7}, and 10^{-8} dilutions were normal.

From these tests it is evident that only slight, if any, decrease in titre of this material occurred during the storage period.

Virus of Acute Meningopneumonitis.—This virus was first described by Francis and Magill (7). The strain, designated California 10, used in these experiments was recovered by Francis and his associates in 1936, presumably from a person ill of an influenza-like disease. The virus produces pneumonia upon intranasal inoculation in ferrets and white mice, and a meningoencephalitis in monkeys and mice upon intracerebral inoculation.

At the time of freezing, this virus had been passed serially through several ferrets and then through 11 series of mice. Whole mouse lungs showing consolidation characteristic of this disease were frozen and stored at −78 C. 3 years later (Apr. 25, 1939) a specimen was thawed and a 10 per cent emulsion in infusion broth was prepared. The emulsion was centrifuged and tenfold serial dilutions were prepared from the supernatant fluid. One series of white mice was inoculated intranasally and another series intracerebrally. 4 mice were inoculated intranasally and 3 mice intracerebrally with each dilution, 0.03 cc. being the individual dose in each instance. The mice were larger than those commonly used. Both aerobic and anaerobic broth cultures of the 10^{-2} dilution were negative at 24 hours, and the aerobic culture showed a light growth of a small Gram-negative bacillus at 48 hours.

Of the mice inoculated intranasally with 10^{-1} and 10^{-2} dilutions, all died between the 5th and 9th day after inoculation and each showed complete or almost complete consolidation of the lungs of a type characteristically produced by this virus. The remaining mice were killed on the 10th day after inoculation and the lungs examined. Those inoculated with 10^{-3} and 10^{-4} dilutions showed extensive consolidation of the lungs, except for 1 mouse in the latter group. Mice inoculated with 10^{-5} and 10^{-6} showed no lung lesions.

Of the mice inoculated intracerebrally all inoculated with dilutions from 10^{-1} to 10^{-6}, inclusive, died between the 4th and 7th day, and in many, death was preceded by paralysis of the extremities. Of the mice inoculated with a 10^{-7} dilution of virus suspension, 2 died on the 6th day and the other showed paralysis of the extremities before the 10th day. Mice inoculated with the next 2 highest serial dilutions remained well.

Although this material was not tested for virulence before freezing, the results of titrations made after storage at −78°C. for 3 years indicate that no appreciable decrease in titre had occurred. The end points observed

here, *i.e.* 10^{-4} by intranasal inoculation and 10^{-7} by intracerebral inoculation, are about the same as those obtained with fresh material.

Virus of Lymphogranuloma Inguinale.—This strain, designated L.C., was recovered from material obtained from the inguinal buboes of a colored female patient who presented the typical clinical picture of lymphogranuloma inguinale of about 5 days duration. The strain was isolated and has been propagated by intracerebral inoculation of white mice. With this strain, using 0.03 cc. of a 10 per cent or 20 per cent suspension of brain tissue as the inoculum, about half the inoculated mice die between the 3rd and 10th day and the remaining mice usually show symptoms indicative of involvement of the brain or meninges.

The material to be tested was prepared from mice inoculated with the 10th animal passage of the virus. Previous passage material had been filtered through a Berkefeld V candle and was bacteriologically sterile. From the brains of mice sick with the disease a 20 per cent suspension in physiological salt solution was prepared, frozen, and stored at −78°C. 10 months later a sample of the frozen material was thawed and tested for virulence. The test material was bacteriologically sterile after 48 hours. Of 4 mice inoculated intracerebrally, 2 died within 3 to 4 days, and the remaining 2 showed neurological changes before the 10th day. Of 4 mice inoculated with a 10 per cent suspension, 2 died within 3 to 6 days, and the 2 survivors showed neurological changes. Of 4 inoculated with a 1 per cent suspension, none died within 10 days, but all became sick by the 8th day and showed difficulty in coordination.

Although titrations of the virus were not made before and after freezing, the foregoing observations indicate that the virus of lymphogranuloma inguinale undergoes no substantial loss of virulence when stored at −78°C., for at least 10 months. The strain has subsequently been successfully propagated from mice inoculated with this frozen material.

DISCUSSION

It is evident from the foregoing experiments that various types of spirochetes and filtrable viruses survive for several years at −78°C., and it is probable that other pathogenic microorganisms would likewise survive for long periods of time. Attention should be drawn to the simplicity of the method of storage, for it can be utilized wherever a constant supply of solid carbon dioxide, which is widely used commercially, is available. Not only have pathogens been stored in this laboratory for a number of years without appreciable loss of virulence, but countless specimens of spirochetes, bacteria, filtrable viruses, and serum have been stored for shorter periods of time. This is the only satisfactory method of preserving the virulence

of the spirochetes of syphilis and of yaws outside a living host, for virulent strains of these organisms cannot be cultivated on artificial media, and they do not withstand desiccation. In the case of other pathogens it has been found convenient to store them at −78°C. particularly for short periods, even though other methods of preservation are available. Storage at this temperature has recently been further simplified by Horsfall (8), who has designed an insulated cabinet in which a temperature of −70°C. to −78°C. can be maintained without the use of a liquid medium such as alcohol.

The available evidence indicates that microorganisms survive equally well at temperatures colder than that of solid carbon dioxide (see review by Luyet and Gehenio (9)), but the latter temperature is more easily maintained under ordinary laboratory conditions. Temperatures of −20°C. or warmer are not satisfactory for the maintenance of spirochetes, but at what point between −20° and −78°C. these organisms begin to be affected adversely is not known, since technical difficulties are experienced in maintaining temperatures within this range. The effect of temperatures of −20°C. or higher will be discussed more fully in the following paper (5).

SUMMARY

1. Observations are reported on the virulence of various types of spirochetes and filtrable viruses after storage at −78°C. for periods up to 3 years.

2. Five specimens of *Treponema pallidum* belonging to 4 different strains, and 7 specimens of *T. pertenue* belonging to 5 different strains were tested after storage for approximately 3 years. With one exception each specimen contained actively motile treponemes, and all specimens were highly pathogenic for rabbits. Many other specimens of these spirochetes stored for shorter periods were also tested with similar results.

3. Relapsing fever spirochetes tested after storage from 6 months to 1 year showed active motility and were virulent for mice.

4. *Leptospira icterohemorrhagiae* was found to be actively motile after storage for 5, 6, and 10 months. The spirochete of rat bite fever, *Spirillum minus*, was virulent for mice after storage for 1 year.

5. The virus of human influenza, PR8 strain, tested after storage for approximately 3 years was fatal to mice in essentially the same dilution as was the same lot of material before freezing. Similar results were obtained upon testing the virus of meningopneumonitis after storage for 3 years. The virus of lymphogranuloma inguinale was pathogenic for mice after storage for 10 months.

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