THE PRESERVATION OF VIRULENT TREPONEMA PALLIDUM AND TREPONEMA PERTENUE IN THE FROZEN STATE; WITH A NOTE ON THE PRESERVATION OF FILTRABLE VIRUSES*

By THOMAS B. TURNER, M.D.

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

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Under natural conditions *Treponema pallidum* and *Treponema pertenue* die soon after removal from a living host. Under laboratory conditions hitherto it has been possible to maintain virulent strains of these organisms only by infection of susceptible animals, since various methods commonly employed in the cultivation or preservation *in vitro* of various bacteria and filtrable viruses have not proved successful when applied to treponemes. It is evident that considerable time and expense are entailed in propagating syphilis or yaws treponemes in animals, particularly if many strains are being maintained. Moreover, this method frequently fails to provide a readily available source of spirochete-containing material for experimental purposes. In this paper will be described a simple method for preserving, *in vitro*, the virulence of *Treponema pallidum* and *Treponema pertenue*, and the application of the same method to the preservation of certain filtrable viruses.

Results of Previous Attempts to Maintain Treponemes in Vitro

At room temperature the motility of treponemes in suspension is usually markedly reduced within an hour or two and rarely does it persist for more than 12 hours. At refrigerator temperature (4–8°C.) the decrease in activity is not so rapid and sluggish motility may be observed after 72 hours. Exceptions to these general statements have been noted, however. Thus Stewart (1) and Lacy and Haythorne (2) claim to have observed motility in specimens after 44 days and 58 and 121 days, respectively. Infectivity for rabbits, likewise, decreases rapidly

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at room or refrigerator temperature. Autopsy or excised syphilitic tissue has been shown to be infective after 24 hours by Lacy and Haythorne (2), after 48 hours by Gregoriev and Jarysheva (3), and after 5 days by Schaffer (4). Recently Rosahn (5), working with excised syphiloma of rabbits' testis which was kept at refrigerator temperature, observed typical lesions of experimental syphilis in rabbits inoculated with 48 hour specimens, but 96 hour specimens produced only slight lesions in 1 of 3 animals. Neither of 2 rabbits inoculated with 7 day specimens exhibited lesions but 1 was infected as proved by lymph node transfer. Neither of the 2 animals inoculated with 14 day, 23 day, and 42 day specimens developed syphilitic lesions, and lymph node transfer was negative in each.

Cultivation of treponemes on artificial media has also failed to provide a satisfactory method for maintaining or propagating organisms which are pathogenic for animals. In a recent review of the subject Kast and Kolmer (6) point out that only in rare instances have treponemes maintained in culture been shown to be virulent for rabbits. These authors likewise failed to grow *T. pallidum* in tissue culture. Essentially the same statements would seem to be applicable to *T. pertenue.*

The Method of Freezing

During the course of attempts to freeze and desiccate treponemes by the technique commonly employed for certain bacteria and filtrable viruses, it was observed that although the treponemes did not ordinarily survive desiccation they were apparently unharmed by the freezing process alone. With this observation as a basis a very simple technique for preserving the virulence of treponemes *in vitro* was devised.

Solid carbon dioxide (dry ice) when placed in 95 per cent ethyl alcohol reduces the temperature of the liquid medium to approximately −78°C., which temperature is above the freezing point of alcohol. This temperature is maintained as long as dry ice is present. When tissue is immersed in this medium, it freezes solid within a few seconds. Any well insulated container may be used for the dry ice-alcohol mixture, but a 12 gallon size container, insulated by vacuum, has been found to be most convenient.[1] Such a container will accommodate about 50 pounds of dry ice, 6 to 8 gallons of alcohol, and still leave space for many specimens. There is little or no evaporation of alcohol. Dry ice must be replenished every 5 to 7 days, approximately 70 pounds per week affording a safe margin. The top of the container is allowed to fit snugly but it is left unsealed in order to permit the escape of gas. The container is kept at room temperature.

The material to be preserved may be frozen as small pieces of tissue or as a

[1] The container used in these experiments was obtained from the American Thermos Bottle Company, Norwich, Connecticut.

suspension in some suitable medium such as saline, broth, or serum. The tissue may be placed in small test tubes or ampoules, the open end of the tube being sealed by heat, or the material may be distributed in 2 cc. to 4 cc. vials which can be tightly stoppered with cork.

For convenient grouping the tubes or vials may be placed in metal boxes with screw caps similar to those used as mailing cases. Perforations should be made in the metal boxes to allow free exchange of alcohol and both vials and boxes should be plainly marked with adhesive tape or with a glass pencil. The metal boxes can be placed in a metal basket or bucket which is allowed to rest on the dry ice in such a manner that all boxes are completely immersed in the alcohol. In freezing and removing specimens the boxes containing frozen material should never be withdrawn for more than a few moments. Heavy gauntlets are useful in handling the cold specimens. Upon withdrawal from the freezing mixture a tube or vial should be opened promptly; otherwise the expanding air will cause the glass to crack. Thawing of the frozen material can be hastened by warming between the hands or by placing the material in an incubator or water bath at 37°C.

The Effect of Freezing on Treponemes

The experiments reported here were devised to show the effect of freezing at −78°C. for various lengths of time upon the morphology, the motility, and the virulence for rabbits of representative strains of *T. pallidum* and *T. pertenue*. The first series of experiments was made with the well known Nichols strain of syphilis treponemes and a strain of yaws treponemes, designated strain YA, which we isolated in Jamaica in 1932. The other strains of syphilis and yaws treponemes employed were all isolated in Jamaica (7).

The material to be studied was usually obtained from the testes of rabbits showing fresh lesions of yaws or syphilis, although in one experiment lymph nodes from infected animals were used. In the case of testicular lesions, except as noted above, the involved portion was divided into small cubes (5 to 10 mm.) of about equal size. One portion of the specimen was then emulsified in physiological saline, the morphology and motility of the treponemes noted under the dark field microscope, and the suspension inoculated into one testis of a normal rabbit as a control upon the virulence of the material before freezing. The other portions were placed in individual tubes and frozen. At various intervals of time a portion was removed from the freezing mixture, allowed to thaw, and emulsified in saline. The morphology and motility of the spirochetes were observed under the dark field microscope and the suspension inoculated into one testis of a normal rabbit in the same manner as the control specimen. Ordinarily from 0.2 cc. to 0.5 cc. was injected. Inoculated rabbits were examined twice weekly or at more

frequent intervals. A positive result was recorded only when the animal presented a lesion characteristic of the one or the other experimental disease and actively motile treponemes were demonstrated in the lesion. In two instances the source material was first emulsified in physiological saline and the resulting suspension used for virulence tests before and after freezing. Thus while the dose for most specimens was only approximately the same at different intervals, in these two specimens (rabbits 2-46 and 2-45) the individual dose for each inoculation was exactly the same (0.3 cc.).

Test inoculations were commonly made at intervals of 14 days, 1 month, 2 months, 4 months, and 6 months, after the material had been frozen, and the virulence of several specimens was tested after 1 year. The first tests made with both syphilis and yaws treponemes may be considered preliminary in nature for the technique as now employed was not then fully developed. Thus in some instances only a few portions of the same specimen were frozen and in several instances a virulence test before freezing was not made. The results with the earlier specimens were not as uniform as with the later specimens.

Effect of Storage at −78°C. Temperature on Morphology and Motility.—When material containing treponemes is withdrawn from the freezing mixture and warmed, thawing is complete within about 5 minutes. A dark field examination at this time shows the treponemes to be normal in shape and size but usually inactive. As the slide is warmed by the heat from the dark field light the spirochetes begin to move, sluggishly at first, but within a few minutes many will exhibit very active motility. In most of the specimens given in Table I the proportion of active treponemes and the degree of motility did not seem to vary significantly, after various periods of time, from that observed before freezing. In some frozen specimens the proportion of active treponemes seemed to be less than before freezing and in a few instances only an occasional treponeme was found to be motile. Frozen specimens from the same lesion, removed from the freezing mixture at different times, occasionally showed variation in motility of the treponemes but there was no observable relation to the duration of the frozen state. Specimens showing poor motility frequently retained their virulence, however, and it is possible that the diminution in motility may not have been an expression of any deleterious effect of the freezing mixture but may have been due to other causes, such as variation in the temperatures to which the treponemes were subjected after thawing. With the exception of the first specimens frozen (Nos. 1-65 and 1-68) all of those examined after 6 months and 1 year showed enormous numbers of actively motile treponemes.

Effect of Storage at −78°C. Temperature on Pathogenicity.—The results of virulence tests made on a number of specimens of *T. pallidum* belonging to the Nichols strain, and of *T. pertenue* belonging to strain YA, both before and at various intervals after freezing, are shown in Table I. Rabbits in which the result is labelled positive in each instance exhibited lesions that were characteristic for syphilis or yaws, respectively, and motile treponemes were demonstrated in the lesion. The incubation period is taken as the day after inoculation on which a clinical lesion first became manifest. In general the results of inoculation of tissues from the same specimen at different intervals after freezing are surprisingly uniform for both the syphilis and the yaws material. The initial orchitis produced by the frozen material was quite as extensive as that produced by the fresh material and differed in no way from that commonly observed after regular passage of the strains of treponemes in question. Among the animals inoculated from frozen *T. pallidum* a lesion failed to develop in only one, and among those inoculated with *T. pertenue* all but 2 developed definite lesions in which treponemes were demonstrated. The incubation period tended to be shorter than is usually observed with these strains, probably because of the large inoculum. Except for the first specimen in each series no definite tendency toward lengthening of the incubation period was noted as the duration of the frozen state was prolonged. Indeed, considering the presence of variable factors such as size of the inoculum, season of the year, and variation in natural susceptibility of different rabbits, the incubation period would seem to be remarkably uniform.

Because of the limitations in space for animals the test rabbits were ordinarily discarded as soon as the initial lesion was well developed, before the period at which metastatic lesions in the uninoculated testis and generalized lesions of the skin, bones, or eye would be expected to appear. Of 6 animals inoculated with *T. pallidum* which had been frozen for 1 year, however, 5 (Nos. 47-81, 47-83, 47-84, 47-86, 47-87) were observed for 90 days or longer after inoculation.[2]

[2] The virulence tests upon specimens which had been frozen for 1 year were made in the laboratory of the Syphilis Division of the Department of Medicine of The Johns Hopkins Medical School. The author wishes to express his appreciation to Dr. Alan M. Chesney, the director of the laboratory, for providing facilities for this work.

TABLE I

Infectivity for Rabbits of Treponema pallidum and Treponema pertenue Which Had Been Maintained at Temperature of −78°C. for Varying Periods up to 1 Year

	Source of treponemes Rabbit No.	Result of virulence test before freezing*	Results of virulence tests at different intervals after freezing*						
			14 days	21 days	1 mo.	2 mos.	4 mos.	6 mos.	1 yr.
Treponema pallidum	1-65	Pos. 17	Pos. 21	Pos. 17	Pos. 21	Pos. 57	Pos. 46	Pos. 33	— —
	1-66	Pos. 17	Pos. 27	Pos. 21	— —	— —	— —	— —	— —
	2-21	— —	Pos. 36	— —	— —	Pos. 41	Pos. 40	— —	— —
	2-23	— —	Pos. 30	— —	Pos. 31	Neg. —	— —	— —	— —
	2-33	Pos. 8	Pos. 25	— —	Pos. 19	Pos. 12	Pos. 7	Pos. 16	Pos. †‡ Pos. 22
	2-37	Pos. 8	Pos. 9	— —	Pos. 10	Pos. 17	Pos. 21	Pos. 13 Pos. 21	Pos.‡ 9 Pos.‡ 9
	2-39	Pos. 12	Pos. 17	— —	Pos. 14	Pos. 7	Pos. 8	Pos. 27	— —
	2-46	Pos. 16	Pos. 32	— —	Pos. 22	Pos. 22	Pos. 18	Pos. 23 Pos. 24	Pos.‡ 22 Pos. 33
Treponema pertenue	1-68	Pos. 28	Pos. 7	Pos. 21	Pos. 27	Pos. 30	Quest.§ 40	Pos. 35 Pos. 28	Quest.§ 40 — —
	1-70	Pos. 14	Pos. 18	Pos. 26	— —	— —	— —	— —	— —
	2-20	— —	Pos. 41	— —	Pos. 29	Pos. 30	Pos. 42	— —	— —
	2-27	Pos. 17	Pos. 19	— —	Pos. 17	Pos. 16	Pos. 14	Pos. 7	— —
	2-29	Pos. 22	Pos. 22	— —	Pos. 20	Pos. 10	Pos. 21	Pos. 23	— —
	2-45	Pos. 28	Pos. 24	— —	Pos. 24	Pos. 28	Pos. 20	Pos. 21	— —
	2-68	— —	— —	— —	— —	— —	— —	— —	Pos. 25
	2-69	— —	— —	— —	— —	— —	— —	— —	Pos. 19

* Numerals denote incubation period in days.

† Clinical lesion masked by non-specific reaction. Motile *T. pallidum* demonstrated, 33 days.

‡ Rabbit developed metastatic orchitis and multiple generalized lesions.

§ Questionable result. Clinical lesion but *T. pertenue* not demonstrated.

All of these animals developed a metastatic orchitis and 4 developed multiple generalized lesions, bone lesions being observed in 4, skin lesions in 4, and keratitis in 1. In other words, the type of disease in rabbits produced by the Nichols strain of *T. pallidum* which had been maintained at −78°C. for 1 year was quite as severe as, and differed in no essential respect from, that commonly produced by fresh material. The same is true of the type of experimental disease produced by *T. pertenue*, although metastatic and generalized lesions are only rarely observed even after the inoculation of fresh material.

Effect of Temperature of −78°C. upon Other Strains of Treponemes.— In order to show that this phenomenon was not confined to the 2 strains of treponemes tested in the foregoing experiment, 5 other strains of *T. pallidum* and 7 other strains of *T. pertenue* were tested in a similar manner before freezing and after having been maintained at −78°C. for 2 months.

These strains were all isolated from human subjects in Jamaica during 1934 and at the time of testing had been propagated in about 6 successive generations of rabbits. It should be particularly noted that the infective material for the syphilis strains was derived from the popliteal lymph nodes of rabbits which at the time showed healing lesions or no lesions at all. Both popliteal nodes were emulsified in physiological saline, one-half of the emulsion being injected into the control animal immediately and the other half being preserved in the frozen state until inoculation 2 months later. The various strains of yaws spirochetes were tested by emulsifying a testicular lesion and employing exactly the same doses for inoculation of the control rabbit and the test rabbit.

The results of the virulence test are shown in Table II. Since no spirochetes were demonstrated in the various inocula of the syphilis group, the influence of freezing on the morphology and motility of the spirochetes which were present could not be determined, but there was obviously no striking decrease in the virulence of the inoculum after freezing. In general the incubation period was slightly longer in those rabbits inoculated with the frozen portion of the emulsion. The tests made with various strains of yaws spirochetes yielded essentially the same results as did those made with strain YA. There was no appreciable alteration in the morphology and motility of the spirochetes after freezing and the disease picture produced in rabbits did not vary significantly from that produced by equal amounts of

fresh material from the same source. In one instance the inoculation of fresh material failed to produce a lesion while inoculation of the same amount of material from the specimen 2 months after freezing gave rise to typical lesions of experimental yaws. This experiment indicates, therefore, that probably all strains of yaws and syphilis spirochetes will survive freezing at −78°C. Moreover, the infectivity of material containing relatively few spirochetes, as well as of material containing large numbers of these organisms, is preserved.

TABLE II

Virulence for Rabbits of Various Strains of Syphilis and Yaws Spirochetes Which Had Been Maintained at −78°C. for 2 Months

Syphilis material, popliteal nodes				Yaws material, testicular emulsion			
Strain of treponemes	Rabbit No.	Virulence test before freezing*	Virulence test 2 mos. after freezing*	Strain of treponemes	Rabbit No.	Virulence test before freezing*	Virulence test 2 mos. after freezing*
S-4	1-80	Pos. 31	Pos. 39	YB	2-31	Pos. 40	Pos. 35
S-5	1-83	Pos. 25	Pos. 33	YC	1-52	Pos. 32	Pos. 36
S-6	1-84	Pos. 13	Pos. 33	YD	1-75	Pos. 37	Quest.† 41
S-8	1-86	Pos. 25	Pos. 36	YE	1-28	Neg.	Pos. 43
S-10	1-89	Pos. 25	Pos. 27	YF	2-43	Pos. 45	Pos. 31
				YH	1-59	Pos. 32	Pos. 60
				YK	2-24	Pos. 34	Pos. 30

* Numerals denote incubation period in days.

† Questionable result. Clinical lesion but *T. pertenue* not demonstrated.

Effect of Other Freezing Temperatures on Treponemes.—Specimens of rabbits' testes rich in treponemes were prepared as in the above experiments. The material in sealed tubes was frozen rapidly at −78°C. and after 2 hours transferred to the ice-making compartment or tray of an ordinary electrically operated refrigerator. By immersing the tubes in 95 per cent alcohol, the tissue was maintained in the frozen state at a temperature approximating −10°C. It was soon evident that treponemes survived at this temperature for much shorter periods than they did at −78°C. and no attempt was made to determine accurately their survival time. The following observation, however, may be noted.

Specimens from 10 different sources used in the previous experiments were frozen at −78°C. and maintained at −10°C., while others were both frozen and maintained at −10°C. In both cases it was noticed that much larger crystals formed in the frozen material at −10°C. than at −78°C. After 3 days, dark field examination showed many normal looking actively motile treponemes, but after 1 week most of the organisms had lost motility and many were shrunken and distorted. Even after 21 days an occasional treponeme showed sluggish motility but it was evident that this temperature was unfavorable to their survival. No difference in this respect was noted between *T. pallidum* and *T. pertenue.* Virulence tests made with *T. pallidum* from 2 rabbits (Nos. 2-39 and 2-46) after the specimens had been frozen at −78°C. and maintained at −10°C. for 14 days gave

TABLE III

Effect of Freezing Temperature versus Maintenance Temperature on Motility and Virulence of Treponema pallidum

Freezing temperature 2 hrs.	Maintenance temperature 2 mos.	Motility of treponemes	Virulence test		
			Rabbit No.	Result	Incubation period
°C.	°C.				*days*
−78°	−78°	Active	T11	Pos.	31
			T12	Pos.	31
−78°	−20°	None	T17	Died	—
			T18	Abscess	—
−20°	−78°	Active	T15	Pos.	28
			T16	Pos.	37
−20°	−20°	None	T13	Neg.*	—
			T14	Neg.*	—

* Observed 90 days.

negative results. While no virulence tests were made with material both frozen and maintained at −10°C., treponemes seem to have been affected adversely to the same extent by this procedure as when freezing occurred rapidly.

It would appear that the damage to treponemes occurs not at the moment of freezing but during the maintenance period.

Similar but somewhat more complete experiments were made by comparing the behavior of treponemes from the same source when subjected to temperatures of −20°C. and −78°C.

From the testis of a rabbit infected with the Nichols strain of *T. pallidum* there was prepared an emulsion rich in treponemes. This emulsion was distributed in vials and the following procedures carried out. One lot of specimens

was frozen at −78°C. and maintained at this temperature for 2 months. Another lot was frozen at −78°C. and after 2 hours transferred to an electrically operated freezing unit which maintains a temperature of about −20°C. A third lot was frozen at −20°C. and after 2 hours transferred to a maintenance temperature of −78°C., and a fourth lot was frozen and maintained at −20°C. Inoculation of the emulsion before freezing produced a typical syphilitic orchitis within the usual incubation period. 2 months after freezing a specimen from each lot was examined for motility of the treponemes and its virulence was tested by intratesticular inoculation of 2 rabbits with 0.3 cc. of the emulsion. The results of these tests are shown in Table III. In both specimens maintained at −78°C. the treponemes were actively motile and produced typical lesions of experimental syphilis in rabbits. No difference was noted between the specimen frozen at −78°C. and that frozen at −20°C. In neither specimen which was maintained at −20°C. were treponemes motile. The virulence tests on the specimens frozen at −78°C. and maintained at −20°C. were, unfortunately spoiled, but rabbits inoculated with material which had been frozen and maintained at −20°C. remained negative for 90 days.

It seems clear that freezing treponemes at −20°C. does not affect the organisms adversely, but maintenance at this temperature over a period of 2 months causes their death.

Effect of Freezing and Desiccation of Treponemes

Tissue containing many syphilis treponemes from 14 different sources was frozen and dried in a glass desiccator. Examination of the dried material after the addition of water showed that the number of treponemes was considerably less than before freezing, all were non-motile and many were distorted. In 5 instances the infectivity for rabbits of dried material from as many different sources was tested with but one positive result. In each case inoculation of material from the same source before freezing gave rise to typical lesions. The quantity of material inoculated after drying was equivalent to from 5 to 15 times the amount of the control inoculum. 3 rabbits inoculated with material 1 day after desiccation remained negative for 3 months and transfer of their popliteal lymph nodes to 2 other rabbits yielded negative results. 3 specimens inoculated 14 days after drying likewise gave negative results. In 1 instance, however, dried material inoculated the day after desiccation gave rise to typical syphilitic orchitis in each of 2 rabbits, 38 and 45 days, respectively, after inoculation, the lesions being rich in treponemes. The incubation period in the control rabbit was 18 days.

It appears that while occasionally *T. pallidum* may survive freezing and desiccation, the technique as employed does not offer a favorable method for preserving these organisms.

The Maintenance of Filtrable Viruses at a Temperature of −78°C.

A number of methods are already available for preserving the virulence of most filtrable viruses *in vitro*. As a rule viruses placed in glycerine and maintained at refrigerator temperature will remain virulent for a number of weeks. Yellow fever virus (8) and the virus of spontaneous encephalomyelitis of mice (9) retain some potency after 160 days when stored at refrigerator temperature. Yellow fever virus has also been preserved in the frozen state at −10°C. for 2 weeks by Sellards and Hindle (10) and at −12°C. for at least 1 month by Sawyer, Lloyd, and Kitchen (11). Moreover, many viruses, as well as certain bacteria, can be preserved for a number of years by freezing and desiccation. With all of these methods, however, there seems to be a considerable fall in titer from that of the fresh specimen. While not the same importance attaches to another method for preserving viruses, the simplicity of the method described above for spirochetes suggests that the procedure may prove to be useful also in the preservation of filtrable viruses. The observations reported here were made on several strains of human influenza virus, 2 strains of yellow fever virus, and 1 strain of the virus of spontaneous encephalomyelitis of mice.[3]

The Virus of Human Influenza.—Studies on human influenza virus were made largely with the PR 8 strain isolated by Francis (12).

From mouse lungs showing extensive consolidation there was prepared a 10 per cent suspension in plain broth. A portion of the fresh material was used for titration, the remainder being distributed in 1 cc. amounts in small vials and frozen in the mixture of dry ice and alcohol. The suspension of mouse lung was bacteriologically sterile. For the purpose of titration tenfold dilutions of the virus were made and 4 to 6 Swiss mice, depending somewhat on the available supply of mice, were inoculated with each dilution. Inoculation of mice was made intranasally under light ether anesthesia, 0.03 cc. being the individual dose employed throughout. Titrations of the virus were made before freezing and 2, 4, and 6 months after freezing. The titer of the virus was determined on the basis of its ability to kill mice and to produce lung lesions within 10 days after inoculation.

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[3] The influenza virus was kindly supplied by Dr. Thomas Francis, Jr., the yellow fever virus by Dr. Max Theiler and Dr. H. H. Smith, and the virus of spontaneous encephalomyelitis of mice by Dr. Theiler.

The results of the several titrations are shown in Table IV. The number of deaths is shown as the ratio of the number dying of pneumonia to the number surviving on the 3rd day after inoculation. All surviving mice were killed on the 10th day and the number showing lung lesions together with those dying of pneumonia are shown as the ratio to the number surviving on the 3rd day. It will be evident that no appreciable decrease in the virulence of the virus occurred during the period of freezing. With a dilution of 1 to 100,000 some deaths occurred in each titration, while lung lesions were produced

TABLE IV

Effect of Freezing at −78°C. on the Virus of Human Influenza

Dilution of virus	Results of mouse inoculation*							
	Before freezing		After freezing					
			2 mos.		4 mos.		6 mos.	
	Deaths	Lung lesions	Deaths	Lung lesions	Deaths	Lung lesions	Deaths	Lung lesions
10^{-1}	—	—	4/4	4/4	6/6	6/6	—	—
10^{-2}	4/4	4/4	4/4	4/4	6/6	6/6	6/6	6/6
10^{-3}	3/4	4/4	4/4	4/4	5/5	5/5	6/6	6/6
10^{-4}	4/4	4/4	4/4	4/4	4/6	6/6	4/6	5/6
10^{-5}	1/4	4/4	3/4	4/4	4/6	6/6	3/6	6/6
10^{-6}	0/4	4/4	0/4	4/4	0/6	5/6	0/6	5/6
10^{-7}	0/4	0/4	0/4	0/4	0/6	0/6	0/6	0/6

* Ratio of number of mice dying or showing lung lesions to number inoculated. Numerator denotes number dying or showing lung lesions; denominator denotes number inoculated, exclusive of number dying before 3rd day.

quite uniformly by a dilution of 1 to 1,000,000. Several other strains of human influenza virus were maintained at −78°C. for periods of from 3 to 4 months. While no titration of this material was made there was no indication of a decrease in the infectivity for mice of the frozen material. Apparently, the virulence of the virus is maintained as well when whole mouse lungs are frozen as when the emulsified lung tissue is first suspended in saline or broth.

The Virus of Yellow Fever.—The titer of 2 strains of yellow fever virus was determined before freezing and after maintenance at −78°C. for 2 and 6 months.

The French neurotropic strain was prepared from mouse brain tissue as a 10 per cent suspension in whole monkey serum. The suspension was bacteriologically sterile. Tenfold serial dilutions were prepared and 6 to 8 Swiss mice were inoculated intracerebrally with 0.03 cc. of each dilution. Surviving mice were observed for 21 days; deaths occurring before the 4th day after inoculation were regarded as non-specific. Portions of the 10 per cent suspension were distributed in vials and frozen at −78°C. After 2 months and 6 months a specimen was removed from the freezing mixture, serial dilutions prepared, and the virus titrated in exactly the same manner as before freezing. The results of these titrations are shown in Table V. Before freezing the virus in a dilution of 10^{-7} killed a majority of the mice inoculated. In the sample tested after 2 months the titer had fallen to 10^{-5} but the titer of the sample tested after 6 months was again 10^{-7}.

TABLE V

Effect of Freezing at −78°C. on Viruses of Yellow Fever and Spontaneous Encephalomyelitis of Mice

Dilution of virus	Yellow fever, mouse brain			Yellow fever, tissue culture			Spontaneous encephalomyelitis of mice		
	Before freezing*	After freezing*		Before freezing*	After freezing*		Before freezing*	After freezing*	
		2 mos.	6 mos.		2 mos.	6 mos.		2 mos.	6 mos.
10^{-1}	—	—	—	8/8	—	—	—	—	—
10^{-2}	—	—	—	7/8	7/8	3/4	—	—	—
10^{-3}	8/8	8/8	—	7/8	7/8	5/6	7/8	7/7	—
10^{-4}	8/8	8/8	6/6	5/8	3/8	3/6	8/8	7/8	4/6
10^{-5}	8/8	4/8	6/6	0/8	0/8	0/6	7/8	7/8	3/6
10^{-6}	8/8	1/8	5/6	0/8	0/8	0/6	3/8	4/8	2/4
10^{-7}	6/8	0/8	4/6	—	—	—	0/8	1/8	0/6
10^{-8}	0/8	0/8	1/6	—	—	—	1/8	—	—
10^{-9}	0/8	1/8	—	—	—	—	—	—	—

* Ratio of number dying to number inoculated with serial dilutions of the viruses. Numerator denotes number dying; denominator denotes number inoculated, exclusive of number of deaths considered to be non-specific.

There is no obvious explanation for the lower titer after 2 months. Another titration at that time yielded substantially the same results.

Another strain of yellow fever virus (strain 17 E) which had been maintained in tissue culture for 222 subcultures, was titrated in a manner similar to the above. Dilutions of the supernatant culture fluid were made with normal salt solution.

The results of titration before freezing and at 2 months and 6 months after freezing are shown in Table V. The titer at each period was essentially the same, namely 10^{-4}.

The Virus of Spontaneous Encephalomyelitis of Mice.—This virus was first described by Theiler (9).

Brains of infected mice were emulsified in plain broth to make a 10 per cent suspension. Several portions of this suspension were frozen at −78°C. From the fresh material tenfold serial dilutions were made and titrated by intracerebral inoculation of Swiss mice with 0.03 cc. amounts. The suspension was bacteriologically sterile. The usual incubation period of this virus is somewhat longer than that of yellow fever virus and varies from 6 to 18 days. Paralysis of the extremities frequently supervenes several days before death of the mouse. Young mice seem to be more susceptible than older mice (9). Surviving mice were observed for 21 days; deaths occurring before the 6th day were regarded as nonspecific. The results of titration of the virus before freezing and 2 months and 6 months after freezing are shown in Table V.

While the end-points of the titrations are not as sharp as those obtained with yellow fever virus, it would appear that no material decrease in the titer of this virus occurred over the 6 month period during which it was being maintained at −78°C.

DISCUSSION

About the beginning of this century it was shown by Brown and Escombe (13) and by Thiselton-Dyer (14) that certain seeds retained the ability to germinate after exposure to the temperature of liquid air (−190°C.) for 110 hours and to the temperature of liquid hydrogen (−250°C.) for 6 hours. Macfayden (15) demonstrated that various bacteria, including *Bacillus typhosus, Corynebacterium diphtheriae,* and *Vibrio cholerae,* were viable after exposure to these same temperatures for 20 hours. A number of years later a technique for the preservation of various bacteria, filtrable viruses, and antibodies by desiccation from the frozen state was developed through the studies of Shackel (16), Swift (17), Sawyer, Lloyd, and Kitchen (11), Elser, Thomas, and Steffen (18), Flosdorf and Mudd (19), and others. In the earlier studies material to be desiccated was frozen in an ice-salt mixture at a temperature a few degrees below 0°C. and it appears that solid CO_2 was first employed for the freezing process prior to

desiccation by Sawyer *et al.* (11). It is evident, therefore, that a variety of pathogens survive exposure to low temperatures for at least a few moments, although apparently no reports have been made before on the effect of below freezing temperatures on organisms of the *Treponema* group.

From the experiments reported in this paper it seems clear that treponemes are adversely affected only slightly if at all by subjection to a temperature of −78°C. while at higher temperatures, −20°C. and −10°C., loss of virulence and death of the organism occur within a few days or weeks. The explanation of this phenomenon is not clear. Considerable experimental work has been done on the effect of freezing as applied to the preservation of fruits and meats for commercial purposes, but the temperature range studied has rarely been below −20°C. and most of the published work deals with the effect upon plant and animal cells of temperatures around or only slightly below the freezing point of water. From this experimental work several theories have been advanced in an effort to explain why damage to cells occurs more readily at temperatures just below the freezing point of water than at lower temperatures.

The first theory proposed was based upon observations of Richardson and Scherubel (20) and Maximov (21). These workers studied cells under the microscope and noted that during freezing at temperatures just below the freezing point of water, ice formed in the extracellular spaces sooner than it did within the cell. Water was thereby extracted from the cell and damage was believed to occur either from mechanical pressure of ice on the cell or else from a rise in concentration of certain salts which are toxic for the cell. Plank, Ehrenbaum, and Reuter (22) showed that the rate of freezing was important, for when tissue was frozen rapidly at low temperatures ice formed simultaneously both within and without the cell and there was little or no detectable migration of water.

Later studies by Moran (23) and Hardy (24) showed that, in addition to the phenomena just noted, the crystalline structure of certain colloids was quite different depending upon whether freezing had been slow or rapid. In slow freezing crystallization progressed from a relatively few centers with attendant distortion of the normal architecture of the colloid, while in rapid freezing crystallization proceeded from many centers, the crystals were much smaller, and upon thawing the normal architecture of the colloid was preserved. Stiles (25), working with solutions of chlorophyll in water, found that when frozen slowly and thawed the chlorophyll flocculated, but when frozen rapidly the solution was unchanged on thawing. Further studies by Moran (23), however, suggest that the explanation of this phenomenon is far from simple. This investigator working with

gelatin gels found that on slow freezing at −19°C. ice formed largely on the surface of the gel from water which had been extracted from the core of the mass. Subjection of the gel to still lower temperatures caused further movement of water from the core to the surface, until the gel reached a concentration of 65 to 66 per cent. Beyond this point no more water was released even when the gel was subjected to a temperature of −190°C. In other words, a certain portion of the water had been "bound" by the colloid and was, in that state, unfreezable. If the rate of freezing was very rapid ice formed evenly throughout the entire mass of colloid material.

The studies just cited were all directed toward determining the physical changes which take place during the freezing process. While it is evident that certain changes do occur at or soon after the moment of freezing it seems clear that such changes cannot explain the observations made upon the effect of freezing on treponemes. *T. pallidum* survived freezing at −10°C. and −20°C. but died during subsequent days or weeks when maintained at these temperatures. This suggests that the damage to the organism which occurs at these temperatures is not due to physical alterations in the cell protoplasm at the time of freezing but to changes which occur during the maintenance period. It has been suggested by Belehradek (26) that death of cells in the frozen state occurs as a result of the accumulation of the products of metabolism, that the metabolic rate is lower at very low temperatures than at higher temperatures. While this would seem to be an attractive explanation of the facts observed in these experiments there is no experimental evidence to support the hypothesis. It is not possible to present in detail all the work bearing upon the effect of freezing on living cells but interesting reviews of the question are given in papers by Maximov (21), Sacharov (27), and Stiles (25, 28), and the annual and special reports of the Low Temperature Research Station of the Food Investigation Board at Cambridge, England.

Whatever the physiological basis of this phenomenon may be, however, it is evident that certain practical uses may be made of the method as described above. The cost of the necessary equipment and of solid carbon dioxide is quite low, and with a suitable container many different strains of pathogens can be maintained for short or long periods of time. The following ways in which the method may

be used are suggested: (*a*) Numerous strains of treponemes or viruses may be maintained in the laboratory with only infrequent animal passage. (*b*) Large quantities of infective material can be made readily available for various experimental purposes. (*c*) In making a series of tests, such as protection tests, over a period of time, one variable factor, the size of the dose of the infecting agent, may be largely eliminated. (*d*) During the course of an explosive epidemic, particularly one due to a filtrable virus disease, many strains of the virus could be recovered, placed in the freezing mixture, and studied in animals at leisure. (*e*) In transporting infective material from the bedside to the laboratory when an appreciable interval must intervene, the material may be transported in the frozen state. Numerous strains of treponemes were thus successfully moved from New York to Baltimore, a 1 gallon size insulated container being used.

SUMMARY

1. A simple method for freezing and maintaining tissue specimens in a mixture of solid carbon dioxide and 95 per cent ethyl alcohol at a temperature approximating −78°C. is described.

2. When frozen and maintained at this temperature *Treponema pallidum* and *Treponema pertenue*, upon thawing, exhibited normal morphology and motility and their virulence for rabbits was not appreciably altered after periods of at least 1 year. This applied to a number of different strains of each organism. The infectivity of material in which treponemes were scant was maintained as well as of material in which they were abundant.

3. At temperatures of −10°C. and −20°C. syphilis treponemes did not survive as long as 2 months. Death of the organism occurred not at the time of freezing but during the maintenance period.

4. Treponemes did not commonly survive freezing and desiccation, although one lot of dried material which contained *T. pallidum* was infective for rabbits 1 day after desiccation.

5. The viruses of human influenza, yellow fever, and spontaneous encephalomyelitis of mice when frozen and maintained at −78°C. showed substantially the same titer after 6 months as before freezing.

6. Certain practical applications of the method are suggested.

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