Lyophilization of Poliomyelitis Virus. Heat Inactivation of Dry MEFl Virus.* (21081)

LISBETH M. KRAFT AND ERNEST C. POLLARD. (Introduced by David Weinman.)

From Department of Microbiology, Medical School and Division of Biophysics, Sloane Physics Laboratory, Yale University, New Haven, Conn.

Few attempts to dry the poliomyelitis viruses have been reported. Landsteiner and Levaditi(1), Römer and Joseph(2), Landsteiner, Levaditi, and Pastia(3) have indicated success in drying infective monkey cords or cord suspensions over KOH, *in vacuo,* and over P_2O_5, respectively. Each of these reports involved testing of the dried material in one monkey, and in no case were virus titrations before and after drying carried out. Epidemiological considerations based on dry virus in nature were indicated principally by Römer and Joseph(2).

In order to measure accurately some of the physical properties of these viruses, *e.g.,* infective particle size as determined by deuteron bombardment(4), it is essential that the material be dried first. This report concerns a method of drying MEFl (Type 2) and Leon (Type 3) poliomyelitis viruses *in vacuo,* as well as the heat inactivation of the former.

Materials and methods. Virus strains. The MEFl baby mouse adapted strain of Casals *et al.*(5) in its 138th passage was used throughout. A large pool of 50% baby mouse brain in water† was prepared, carefully homogenized, divided into aliquots, frozen and stored at −70°C until needed. It was diluted before drying to a 10 or 20% suspension with any of the media to be described. Spinal cords (5% suspension) from adult mice infected with the mouse adapted Leon virus(6, 7) in its 90th passage were also used.‡ *Virus titrations.* These were done in adult Swiss albino mice by the intraspinal technic of Habel and Li(8). Ten-fold virus dilutions were prepared in saline and 8 mice per dilution were inoculated with 0.02 ml each. They were observed 10 days. The PD_{50} was estimated by the method of Reed and Muench (9), and the titers given below were calculated per inoculum. *Lyophilization.* The material to be dried was shell frozen in small Pyrex ampules in 0.2 ml amounts. Evacuation was effected by a pump with the aid of a condenser immersed in a mixture of methyl cellusolve and dry ice, the entire set-up being kept at room temperature. With the small volumes used, drying was complete in 6 to 8 hours, or 1½ times the time required for all visible moisture to disappear from the outside of the tubes. Later it was found that the procedure was aided by keeping the material cold while it was drying. Thus a mixture of crushed ice and rock salt surrounded the tubes. After drying was complete, dry air was admitted and the tubes were sealed. In no case was it attempted to seal the tubes *in vacuo. Lyophilizing media.* In checking routine bacteriological media for their possible protective effect on dry poliomyelitis virus, it was found that peptone water (1% Bacto-peptone§ and 0.5% NaCl in water) and Na thioglycollate broth§ were relatively successful as compared with water or saline alone. Various concentrations in water of peptone (from 2 to 30%) at pH 7, Na thioglycollate (2.5 and 5%) at pH 8, 0.5% gelatin, 3% cysteine • HCl neutralized to pH 5 with NaOH, and beef heart infusion broth were prepared. Each of these was added to the 50% baby mouse brain in a volume that yielded a 10 or 20% brain suspension. When Leon virus was dried, 1 part of a 10% cord suspension that had been frozen and thawed twice with subsequent centrifugation was added to 1 part of 30% peptone. *Reconstitution of the dry virus.* The dry material was reconstituted either in water or 30% peptone

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† Demineralized water was used throughout.

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§ Difco Laboratories, Detroit, Mich.

TABLE I. Results of Drying MEF1 Poliomyelitis Virus in Various Media.

% baby mouse brain	Drying medium*	Dried in cold‡	Reconstituting medium	Virus titer† Before drying	Virus titer† After drying	% virus survival
10	10% peptone	Yes	Water	5.9	5.4	32
20	30% "	"	"	6.0	5.5	32
10	5% Na thioglycollate	"	"	5.0	4.5	32
10	5% peptone	"	"	5.9	5.0	13
10	10% "	No	"	5.7	3.5	.6
20	10% "	"	"	5.9	4.4	3
10	Peptone water	Yes	"	5.7	4.6	5
10	*Idem*	No	"	6.0	4.3	2
10	Thioglycollate broth	"	"	6.0	4.3	2
10	0.5% gelatine	Yes	"	5.7	3.5	.6
10	Cysteine	No	"	5.3	3.4	1.3
20	"	"	"	5.3	4.0	5
10	Water	"	"	6.0	<3.0	<.1
20	"	"	"	5.9	<3.0	<.1
20	"	Yes	"	5.9	3.5	.4
10	Saline	No	"	6.0	<3.0	<.1
10	Infusion broth	"	"	6.0	<3.0	<.1
20	Water	Yes	30% peptone	6.0	5.0	10
10	"	"	*Idem*	6.0	5.0	10

* See text for details. Original concentrations are given.

† Reciprocal of log PD_{50}.

‡ "Yes" indicates material kept cold throughout drying procedure. "No" indicates material allowed to reach room temperature during drying.

so that the final brain concentration was 10%. Either the reconstituted material underwent a single cycle of rapid freezing to –70°C followed by rapid thawing, or reconstitution was allowed to take place at 4°C for 6 hours. In any one experiment where drying technics were being compared, only one of these methods was used. *Heating of dry MEFl virus.* The material to be heated was dried as 20% brain suspension in 18% peptone (final concentrations). After sealing, the tubes were held at –70°C until they were heated. Control (unheated, dry) material was kept at –70°C until it was reconstituted for titration. Duplicate tubes were heated at 10° increments from 0° to 80°C for 20 minutes. Other duplicate series were heated at 40°C, 50°C, and 60°C for various times. The entire tube was immersed in a water bath whose temperature varied ±1°C. At the end of the holding period it was immediately plunged into dry ice-alcohol and kept at –70°C until it was reconstituted in water and titrated in mice. The contents of duplicate tubes were pooled for titration.

The results of one experiment are included below in which virus was dried at room temperature in summer (about 35°C) and stored immediately thereafter in sealed ampules at 37°C for 70 days. Titrations were carried out immediately after their removal from the lyophilizer, and at 7, 13, and 70 days thereafter. The time elapsing between the disappearance of external ice and water from these tubes and titration of the virus before storage was approximately 2 hours.

Results. Table I shows the titers before and after lyophilization of MEFl virus in various media at room temperature and in the cold. It can be seen that % survival was best when the material, while drying, was kept cold in the presence of peptone or Na thioglycollate with reconstitution in water, or when it was dried in water and then reconstituted in 30% peptone. The remaining media gave intermediate to very poor results when water was used for reconstitution.

Not included in Table I are the results of one experiment in which Type 3 (Leon) virus was dried in the cold as 5% mouse cord in 15% peptone. Before drying, its PD_{50} was $10^{-2.6}$. After desiccation and reconstitution in water, the titer was $10^{-1.6}$.

The results of heating the dry MEFl virus are presented in Fig. 1 and 2. Fig. 1 indicates that holding the preparations for 20 minutes

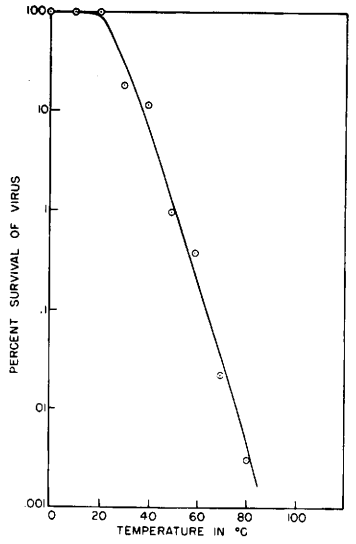

FIG. 1. Heat inactivation of dry MEF1 virus at constant time (20 min.).

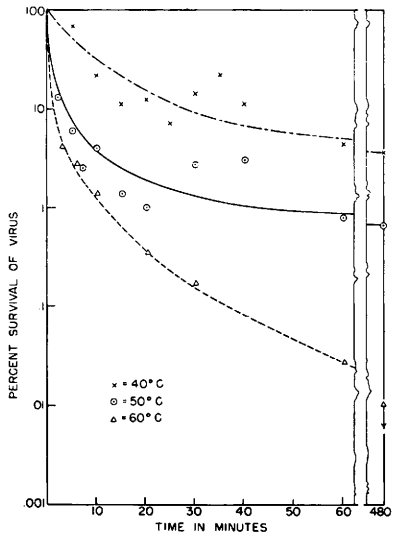

FIG. 2. Inactivation of dry MEF1 virus at 3 temperatures. Arrow at extreme right of the 60°C curve indicates virus survival of less than 0.01%.

at 20°C and below caused no demonstrable activity to be lost. Between 20° and 30°C degradation began and then followed a reasonably straight line when log of % survival is plotted against temperature. When temperature was held constant (Fig. 2) and time varied, the curves derived are no longer exponential. Instead, they tend to flatten out with increasing time. In the single trial in which dry MEF1 virus was held at 37°C for 70 days, we assume that, had the material been cold during drying instead of at about 35°C, the control titer would have been comparable with that of other controls, *viz.*, about 10^{-5}, and that the titer, $10^{-3.5}$, obtained just before storage at 37°C represented roughly 3% of the original dry activity. At any rate, after 7 days at 37°C, the titer was $10^{-2.7}$, at 13 days, $10^{-1.8}$, and at the end of 70 days it was $>10^{-0.7}$, 7/7 mice becoming paralyzed and dying when this concentration alone was tested.

Discussion. The results of our experiments indicate that lyophilization of Type 2 and Type 3 poliomyelitis viruses can be accomplished with little loss of infectious activity. This is in contradiction to the current belief that poliomyelitis virus does not survive desiccation (10). If drying is carried out at a low temperature, and if the virus is lyophilized *or* reconstituted in a suitable medium, there is little loss of infectivity. The results further indicate that peptone, Na thioglycollate, and cysteine afford good, or reasonably good, protection for the virus, thus suggesting that mild reducing conditions (as found in –SH compounds) may be necessary to prevent loss of infectivity. This appears to be true even when the temperature is kept low during desiccation. Peptone, containing a variety of amino acids, probably meets the requirement in the same way. Search for the precise mechanism of protection might be undertaken with highly purified virus preparations, but this has not yet been attempted.

The salutary effect of the low temperature during drying is explained by the heat inactivation curve of Fig. 1, for at the usual room temperature, 20°C to 30°C, inactivation begins to appear even at the end of 20 minutes. Thus, when all external ice and moisture have gone from the lyophile tubes and the last traces of water are being removed from within, the entire contents are subjected to room temperature for several hours. During this time a variable amount of heat inactivation can occur.

Because the curves in Fig. 2 are not straight lines, the line drawn in Fig. 1 cannot be taken too seriously. Heating at 40° and 50°C (Fig. 2) brings out the fact that a heat re-

sistant virus population is present in our preparations, for there is little or no difference between the % survival at 60 minutes and 480 minutes in either case. At 60°C, however, this heat resistant segment itself begins to decay, for the curve continues downward although it still does not seem to be a straight line, and at 480 minutes no virus could be detected at 0.01% survival. In addition, the fact that infective virus was still present in the preparation held at 37°C for 70 days speaks for the existence of a heat resistant population. Experiments are in progress to study the heat resistance of MEFl virus and to ascertain whether or not the same inhomogeneity with respect to heat stability applies to the mouse adapted Type 1 and Type 3 viruses.

Although in such a mixed virus population the analysis of the thermal constants is not of precise significance, the figures obtained by assuming that two varieties are present are of interest. The fast inactivating component has a heat of activation of 28,800 calories per mole and an entropy of activation of +21 calories per mole per degree. The slow component has a heat of activation of 20,800 and entropy of −10 in the same units. This last indicates that in the dry condition the slow component is capable of withstanding a wide range of temperatures without becoming inactivated.

The epidemiological implications of these results are obvious if they are applicable to poliomyelitis viruses in general; and the possibility that dry virus may play some part in the dissemination of disease cannot be put aside. If conditions in nature are adequate for the protective drying and/or reconstitution (in the host?) of the dry virus, this becomes more than a possibility. Even though full activity has not been retained in the material dried as outlined above, it has been shown that 10 to 30% of the original activity can be recovered after drying and that, in addition, when exposed to air, a segment of the viral population is quite heat resistant and persists for some time. It remains to be seen, however, if desiccation of natural virus-containing materials such as feces, sewage, saliva, naso-pharyngeal exudates, etc., will yield similar results, and it would be important in this regard to gather information on the degradation of the dry virus during several cycles of drying and reconstitution, ultraviolet inactivation, temperature variation applied to a single dry preparation, and any other conditions that simulate natural climatological events. We have simulated nature in only one respect, *i.e.* the dry virus was heated in the presence of air.

Summary. 1. Data are presented showing that the MEFl (Type 2) baby mouse adapted strain of poliomyelitis virus can be lyophilized with little loss of infectious activity. The same appears to be true of the Leon (Type 3) mouse adapted strain. 2. Heating the dry MEFl virus indicates that, although part of the viral population appears to be relatively heat labile, a measurable proportion of the virus particles is quite resistant to heat. 3. The epidemiological implications of the possible presence of dry poliomyelitis virus in nature are discussed.

1. Landsteiner, K., and Levaditi, C., *Compt. Rend. Soc. Biol.*, 1909, v67, 787.

2. Römer, P. H., and Joseph, K., *Münch. Med. Wschr.*, 1910, v57, 945.

3. Landsteiner, K., Levaditi, C., and Pastia, C, *Ann. Inst. Pasteur,* 1911, v25, 805.

4. Pollard, E. C., and Kraft, L. M., in preparation.

5. Casals, J., Olitsky, P., and Anslow, R. O., *J. Exp. Med.*, 1951, v94, 111.

6. Li, C. P., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 233.

7. Li, C. P., and Schaeffer, M., *ibid.*, 1953, v82, 477.

8. Habel, K., and Li, C. P., *ibid.*, 1951, v76, 357.

9. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

10. Hammon, W. McD., in *The Dynamics of Virus and Rickettsial Infections,* Blakiston, N. Y., 1954, p188.

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