THE EFFECTS ON BIOLOGICAL MATERIALS OF FREEZING AND DRYING BY VACUUM SUBLIMATION

II. Effect on Influenza Virus*

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In the preceding paper of this series we have described an efficient and versatile apparatus for freeze-drying by vacuum sublimation. This apparatus makes it possible to dehydrate biological materials at low temperatures and, by the use of ionization gauges, to determine the end-point of dehydration of material maintained at temperatures ranging down to −80°C. It is so designed that different samples of the same material can be subjected simultaneously to various experimental conditions.

Time, rate, and degree of drying have been shown to have a marked influence on the physical properties of lyophilized biological materials other than viruses by Elser, Thomas, and Steffen (1); Greaves and Adair (2); Flosdorf, Hull, and Mudd (3); Greaves (4) and others. Proom and Hemmons (5) have published working details for the freeze-drying preservation of a collection of more than 1500 strains of bacteria. Various methods of freezing, the degree of drying, and the effect of storage were tested by viability counts. Recently, Hutton, Hilmoe, and Roberts (6) have reported on the effects of these factors on the quantitative survival of *Brucella abortus*. Similar studies dealing with the survival of viruses have not been reported.

The present investigations deal with the quantitative survival of influenza virus suspensions, after repeated freeze-thaw cycles, freezing at various speeds, storage in the frozen state at various temperatures, and vacuum sublimation at various temperatures following different types of preliminary treatment. Subsequent investigations are planned which will be concerned with the effects of these factors on the survival of complex cells.

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Material and Methods

The virus used was the PR8 strain of influenza A virus, which has been maintained in fertile eggs for several years in this laboratory. In the intervals between egg inoculations, the virus was maintained by storing pooled allantoic fluids in a deep-freeze box at −40°C. The apparatus used has been described in the preceding paper of this series. Infectivity titres were determined by making 10-fold dilutions in physiological saline, and injecting 0.1 ml of each dilution into the chorioallantoic sacs of each of 5 eggs (7). The 50 per cent end-points were calculated by the method of Reed and Muench (8).

RESULTS AND DISCUSSION

Effects of Repeated Freeze-Thaw Cycles. This experiment was carried out both for its intrinsic interest and to determine whether it would be possible to use a single large volume of pooled allantoic fluids for several experiments carried out on successive days.

Allantoic fluids from a number of infected eggs were pooled, and the infectivity titre of the mixture was determined. Two test tubes, each containing 2 ml. of this suspension, were placed in the deep-freeze box at −40°C. As soon as the solid state was reached, both tubes were removed, and their contents allowed to melt at room temperature, this process requiring about 20 minutes. The infectivity titre was determined by using 0.1 ml. of each suspension.

From this point on, one tube was used for determining the effect of repeated freeze-thaw cycles, while the other (the control) was maintained in the liquid state at 0°C. The melting phase of each freeze-thaw cycle was carried out at 0°C. After each freeze-thaw cycle, 0.1 ml. of the fluid was withdrawn to determine the infectivity titre, the remainder being returned to the deep-freeze box at −40°C. At the same time 0.1 ml. of the control fluid, stored at 0°C., was used for titre determination.

After 5 freeze-thaw cycles, the infectivity titre fell from $10^{-8.6}$ to $10^{-0.8}$ (Fig. 1) while that of the control fluid fell only to $10^{-6.5}$. The loss of titre was relatively slight after the first cycle, increased rapidly with the next 3 cycles, but tended to level off at the 5th cycle, suggesting an asymptotic relationship. It may be concluded that a marked loss of infectivity titre resulted from the mechanical process of freezing and thawing, as distinct from the loss which occurred as a result of storage at 0°C.

Kyes and Potter (9) found that a small number of avian tubercle bacilli survived 25 cycles of freezing and thawing. There was marked reduction in the ability of these surviving organisms to multiply in culture or in a host. The survival curve for the separate cycles was not determined. Lennette and Smith (10) reported that cyclic freezing and thawing of St. Louis encephalitis virus caused a slow diminution of titre. Proom (11) has suggested that the death of bacteria frozen in a liquid suspension may be due to puncture of their cell walls by extracellular ice crystals, and that species' differences in survival are the result of differences in the strength of cell walls.

Assuming that the death of influenza virus particles is caused by a similar

mechanism, a possible explanation of the type of survival curve which we obtained may be advanced on the basis of known physicochemical phenomena (12). When a liquid is first frozen, supercooling usually occurs, and solidification does not occur until seed crystals have formed. Supercooled liquids freeze rapidly, once the process starts, but because only a few nuclei are present, the ice crystals are large. The crystalline nuclei are not destroyed by thawing unless the temperature is raised to 50–95°C. (13). With subsequent freezings from lower temperatures, the intact seed crystals prevent super cooling, and freezing

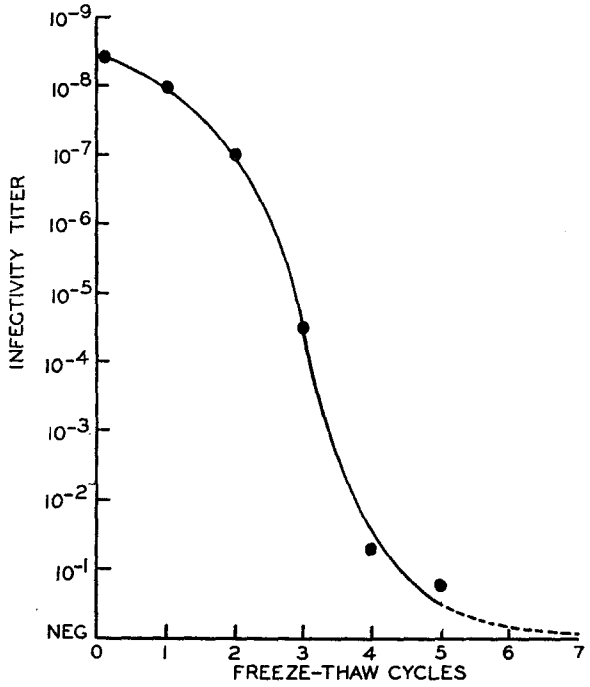

FIG. 1. The effect of cyclic freezing and thawing on the infectivity titer of influenza A virus suspension.

takes place slowly, with the formation of more numerous crystals of a smaller size. The relatively small loss of titre after our first freeze-thaw cycle may be due to the fact that the larger crystals were so arranged that large interstices, which functioned as safety zones, occurred between them. With subsequent cycles the smaller crystals, being in closer apposition, would tend to cause more mechanical injury to trapped particles. Another factor might be the summation of individually sublethal injuries. Possibly, also, the levelling off of the curve at the 5th cycle may be due to the smaller number of live virus particles present.

We cannot of course exclude the possibility that ice formation within the virus particle may play a role in its destruction. Kiermeier (14) has reported that repeated freezing and thawing caused the precipitation of enzymes in extracts of living materials, and this phenomenon also may contribute to the loss of viable virus particles in our experiments.

Effects of Speed of Freezing and Storage Temperature.—The purpose of these experiments was to determine the effect on the survival of virus of (*a*) rates of temperature change, (*b*) storage in the frozen state, and (*c*) temperature changes while in the frozen state.

Freshly harvested pooled allantoic fluid was used, and the volume of fluid subjected to each experimental condition was always 0.2 ml. The different rates of temperature change were obtained by varying the masses to be cooled and the differentials between the initial and final temperatures. The most rapid rate of temperature change resulted when the virus suspension at room temperature was expelled as droplets into a test tube cooled to and maintained at −190°C. The slowest rate of temperature change was brought about by pipetting the suspension at 0°C. into a test tube chilled to 0°C. and cooling slowly to −10°C. Rates intermediate to the above extremes were obtained by pipetting the fluid into test tubes at room temperature or 0°C. and then placing these tubes at −10, −20, −40, −60, or −80°C. Other intermediate speeds of cooling were obtained by expelling droplets of the fluid into test tubes cooled to and maintained at the foregoing temperatures. Infectivity titres were determined on suspensions of virus thawed shortly after the terminal temperatures were reached and after storage at these temperatures for several weeks. Other suspensions were frozen in a similar manner to the above and then stored at higher temperatures for several weeks before determining infectivity titres. The storage temperatures were 0, −10, −30, −40, −60, and −80°C. Thus materials frozen at −190°C. were stored at 0, −10, −30, −40, −60, and −80°C. while suspensions frozen at −40°C. were stored at 0, −10, and −30°C.

The type of solidification occurring under different conditions (large crystals, small crystals, and vitrification) was determined by study of the frozen virus suspensions *in situ*, by means of a microscope equipped with a low power lens and polaroid discs.

The infectivity titres of virus suspensions rapidly cooled to temperatures of −40°C. or lower and thawed shortly after terminal temperatures were reached did not differ significantly from those of the control suspensions. Similar results were obtained with suspensions slowly cooled to −80 or −190°C. Significant decreases of infectivity titre resulted when virus suspensions were rapidly cooled to temperatures above −40°C. or slowly cooled to temperatures above −80°C.

Significant changes in infectivity titre were not found to occur as a result of storage for 3 weeks at −40°C. or at lower temperatures; at temperatures above −40°C., definite loss of titre occurred during the storage period. These statements hold true regardless of the temperature to which the material was originally cooled. Fast cooling to −80 and −190°C. caused vitrification of the virus suspensions, as determined by examination with polarized light. Slow cooling to −190°C. resulted in partial vitrification. The rates of temperature

change brought about by the other terminal temperatures used resulted in the formation of ice crystals of varying size and complexity.

The literature on the biophysics of low temperatures contains many reports of the effects of cooling to subzero temperatures on the survival of cells, tissues and organisms (15–20). In general it has been found that the temperatures dangerous to living cells lie between −4 and −40°C. Intra- and extracellular water form crystals when cooled to temperatures within this range. These crystals have been reported by some investigators to cause irreversible injury to cells by mechanically rupturing cellular membranes. Others believe that cellular damage caused by the freezing process results from the increased salt concentrations in the intracellular fluids. Many of the deleterious effects of freezing have been avoided by ultrarapid cooling, partial dehydration of cells and tissues, and by the use of protective agents. Ultrarapid cooling (a temperature change of several hundred degrees per second) lowers the temperature of the water of cells and tissues through the critical zone (−4 to −40°C.) with a velocity sufficiently great to prevent the formation of crystals. Partial dehydration may remove enough of the free water of the cells so that little, if any, crystalline ice can be formed. The action of protective agents (ethylene glycol (17, 19, 21), glucose (17, 21), polysaccharides (22), and proteins (21)) appears due to the ability of these substances to penetrate cells and to render some of the intracellular water unfreezable or to change the type of crystals formed.

In some of our experiments there was no demonstrable loss of titre, even though neither ultrarapid cooling or partial dehydration was used. In this respect, influenza virus appeared to differ from more complex cells which can survive the increased density occurring with vitrification but not the molecular rearrangements brought about by crystallization.

Lovelock (19, 20) using hemolysis as a criterion for estimating cell damage, found the critical temperature, below which little injury occurred, to be −40°C. He also observed that maximum damage does not occur at temperatures below −40°C., when large amounts of ice are present and salt concentrations would be expected to be highest, but at temperatures between −40 and −4°C. when there is still sufficient unfrozen fluid to maintain the red blood cells freely in suspension. Thus it appears that the formation of ice and the other known physical changes that accompany the freezing process do not account entirely for cellular damage occurring at temperatures above −40°C.

The effects of low temperatures and the frozen state on the kinetics of enzymes and enzyme systems have been investigated to a limited extent (23). Invertase (24–26), pancreatic lipase (14, 27, 28), catalase (14, 29, 30), protease (27, 31), pectic enzymes (24), and peroxidase (32) have been found to be active in the frozen state. The data indicate that even though enzyme action continues in the frozen state, ice formation has a markedly retarding effect. The

degree of reduced activity is not the same for all enzyme-substrate systems. Therefore, low temperatures may result in imbalances in the integrated stepwise enzymatic reactions. These imbalances may lead to the accumulation of metabolic intermediates.

Assuming that the influenza virus possesses some enzymatic activity, the above facts may explain in part the losses in infectivity titre which we found to occur on storage of virus suspensions at temperatures above −40°C.

Our observations are also in harmony with those made by others, using more complex biological entities. Luyet and Hartung (33) have reported that under certain conditions, the vinegar eel (*Anguillula aceti*) can be revived after solidification in liquid air. It was found, however, that exposure of the frozen worms to temperatures from −39 to −5°C. for 1 minute was always fatal. Exposure to temperatures from −43 to −50°C. for 30 minutes did not prevent revival. The mortality of the eels increased rapidly when the temperature of exposure passed from −43 to −39°C. Lovelock (19, 20) has shown that red blood cells, although not seriously injured by rapid freezing at −80°C. and rapid thawing to room temperature, suffer extensive damage if they are first cooled to −80°C. and then stored at a temperature of −30 to −40°C. for 30 minutes.

It has recently been found that raising the temperature of crystalline water above the terminal temperature, without permitting thawing, brings about growth of the crystals. Thus the increased damage observed when biological entities are stored at temperatures above −40°C. after preliminary cooling at a lower temperature is probably due to changes in crystal size (34), sudden decrease in salt concentration, and modified enzyme activity.

Effects of Preliminary Treatments and Sublimation Temperatures.—

Pooled allantoic fluids from 5 infected eggs were used for each experiment (Fig. 2). The decay in titre resulting from repeated freeze-thaw cycles (Fig. 1) made it necessary to use a separate pool for each experiment. The infectivity titre of each pool was 10^{-7} or higher. The material was stored at −40°C. until used. After thawing at room temperature, some samples (0.1 or 0.2 ml.) were used to determine the titre; other aliquot samples were transferred to sterile lyophilization vials. Thus comparisons were always made between the titres of the original material after one freeze-thaw cycle (the controls) and the same material after one freeze-thaw cycle plus a cycle of lyophilization under a particular set of experimental conditions.

When rapid freezing of the virus suspension was desired, the tubes were cooled to the preliminary temperatures (−190, −80, −60, and −30°C.) by immersing them in Dewar flasks containing appropriate cooled liquids or sludges. The virus suspension was then added with a calibrated pipette, the end of which was drawn out to produce fine drops. For slow freezing the virus suspension was placed in the lyophilization tubes and the tubes were rotated as they were lowered at an angle into the cold bath. This produced a thin shell of material on the lower portion of the tubes. The tubes remained in the cold baths at the preliminary temperatures until placed on the secondary manifold and until a vacuum of 5×10^{-2} mm. of Hg was established within the vacuum line. The flasks were then replaced

by others containing cooled liquids or sludges at the temperatures to be used for dehydration.

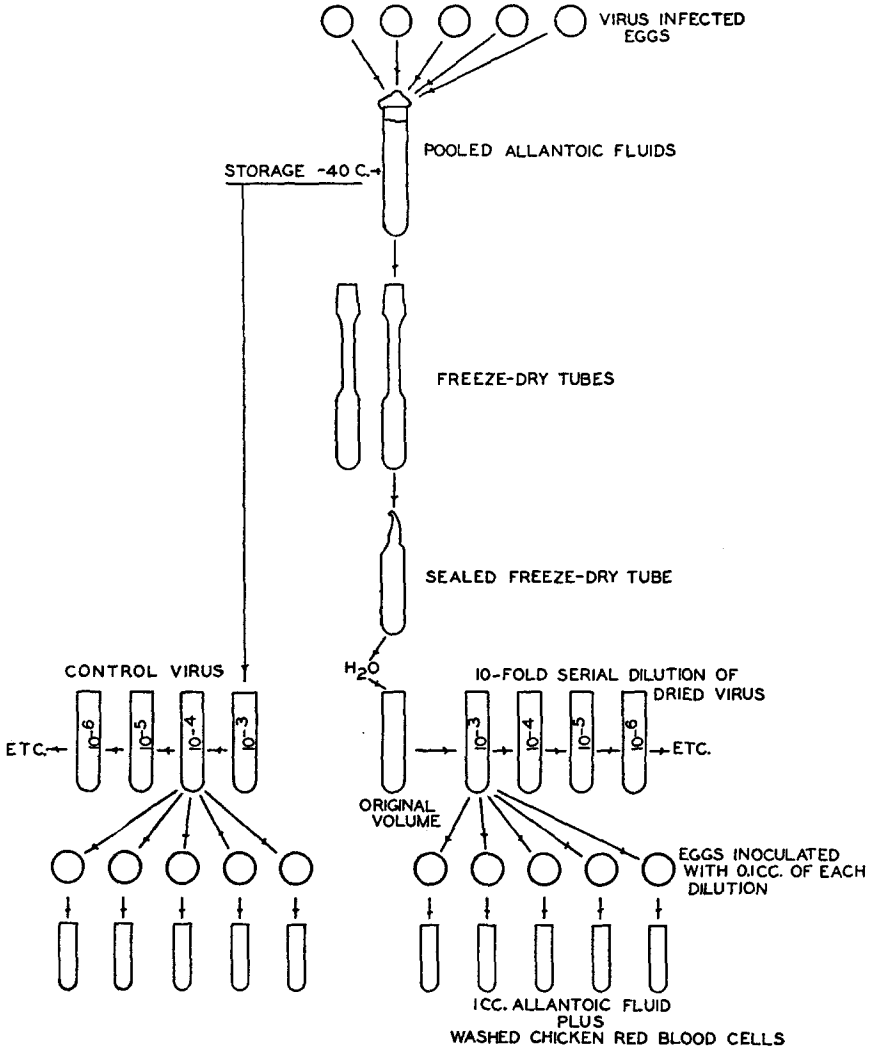

FIG. 2. General plan of experimental procedure.

Some virus suspensions were preliminarily frozen by "snap freezing". This process depends upon a rapid lowering of pressure within the vacuum system. The high rate of water evaporation brought about by the lowered pressure results in a rapid cooling of the liquid phase of the suspension and eventually in the formation of ice. Immediately after the formation of ice the lyophilization tubes were immersed in Dewar flasks containing liquids or sludges at the temperature to be used during vacuum sublimation.

For each set of experimental conditions, 2 or 4 duplicate tubes of virus suspension were used. Upon completion of dehydration in each group of tubes, these tubes were sealed as described in Paper I of this series. The sealed tubes were then stored at −40°C. until dehydration was complete in all tubes used for a particular experimental run. All tubes were then opened, the dried materials reconstituted to original volumes with distilled water, and the titres of the reconstituted virus suspensions were determined (Fig. 2). (Previous experiments had shown that no significant changes in titre occur when dried material is stored at −40°C., even though dehydration may have been carried out at higher temperatures.) Each experiment was carried out at least 5 times in order to assure statistical significance.

TABLE I

The Effects of the Preliminary Treatment and of the Sublimation Temperature on the Preservation of Influenza Virus

Preliminary treatment*	Sublimation temperatures					
	20°C.	0°C.	−10°C.	−30°C.	−60°C.	−80°C.
Snap frozen from 20°C.	−4.0‡	−2.5‡	−3.2‡	−3.7‡	−2.4‡	−2.7‡
Snap frozen from 0°C.	−4.0	−2.3	−3.0	−3.5	−2.3	−2.0
Slow frozen at −30°C.	−3.0	−2.0	—	−2.7	−2.5	−2.6
Slow frozen at −60°C.	−3.0	−1.2	—	−3.0	−2.9	−1.9
Slow frozen at −80°C.	−2.0	−0.5	—	−2.4	−2.0	−0.6
Slow frozen at −190°C.	−1.5	+0.8	—	−1.4	−1.0	0.0
Fast frozen at −30°C.	−3.0	−1.4	—	−2.8	−2.5	−2.4
Fast frozen at −60°C.	−2.2	−0.6	—	−2.4	−1.7	−0.8
Fast frozen at −80°C.	−1.5	+0.6	—	−1.7	−1.0	+0.2
Fast frozen at −190°C.	−1.2	+0.6	—	−1.4	−0.7	+0.8

* For explanation of terms see text.

‡ Figures indicate loss (−) or gain (+) in infectivity titre (expressed in logs) at the end-point of vacuum sublimation as compared with non-sublimated controls (see text).

The effects of varying preliminary treatment and sublimation temperatures on infectivity titre are shown in Table I. The use of stock virus suspensions from several different groups of eggs for repeat experiments, with consequent minor variations of the ID_{50} made it necessary to determine the central tendency of the infectivity titres. The exponential character of the test for the ID_{50} led us to use the geometric mean of the titres. This was obtained by adding the exponents of the infectivity titres and dividing by the number of exponents summed (arithmetic mean of the exponents). The figures in Table I show the differences between the means of the control virus suspensions and the means of the lyophilized virus suspensions. Calculations based on the data of Knight (41) have shown that a difference in logs of 0.6 is statistically significant at the 95 per cent probability level (7, 42). A plus sign before the difference listed indicates that the treated suspensions showed a higher mean infectivity

titre than the controls and a negative sign that the treated materials had a lower titre than the controls.

The manner of handling the virus suspensions before dehydration and the temperature of the suspensions during sublimation, in most instances, altered the infectivity titre. With slow preliminary cooling to −80°C. and subsequent dehydration at 0°C., the infectivity titre was not significantly different from that of the control suspensions. Similar results were obtained when preliminary cooling was carried out rapidly to −80°C., or slowly to −190°C., with subsequent dehydration at −80°C.

With preliminary rapid cooling of suspensions at −80°C. and both fast and slow cooling at −190°C. a probably significant increase of the infectivity titres was found after vacuum sublimation at 0°C. Such an increase was also found for suspensions cooled rapidly to −190°C. and dried at −80°C. All other combinations of cooling and sublimation temperatures resulted in significant losses in infectivity titre.

The greatest loss of infectivity titre as a result of preliminary treatment, for every sublimation temperature employed, was observed in virus suspensions frozen by "snap freezing." In considering the effects of sublimation temperatures, it will be noted that the greatest declines of infectivity titre were found in the suspensions sublimated at +20°C.; the smallest declines at 0°C., and −80°C. At sublimation temperatures of −10, −30, and −60°C. the losses in infectivity titres were intermediate.

In general, declines in infectivity titre for all dehydration temperatures were smallest with preliminary cooling to −190°C. The sequence of infectivity titre decline as related to the preliminary cooling temperature was as follows: −190, −80, −60, −30°C. Although the drop in titre of the slowly frozen virus suspension was greater than that of the rapidly frozen suspensions, the above order of infectivity titre loss was observed in both groups.

Following all manners of preliminary treatment the smallest losses of infectivity titre were invariably associated with sublimation temperatures of 0 and −80°C. Suspensions dehydrated at +20, −30, and −60°C. showed significantly greater losses in infectivity titres, and in general, the losses at these temperatures were quantitatively comparable.

In many of our experiments, the infectivity titre losses after freezing and drying were greater than would be expected from the freezing factor alone. In general, drying from the vitreous state gave better preservation of viability than did drying from the crystalline state. The basis for this observation may be physical differences in the molecular arrangements of the two states (12, 35–37). These differences could lead to differences in the energy content of the virus particles or to differences in loss of water from virus proteins during dehydration of virus suspensions at low temperatures.

The water molecules of ice crystals are arranged in a highly ordered lattice

form and are consequently in equilibrium. For the crystalline state the lattice configuration is the unit cell, which is, therefore, the fundamental entity of this state. The water molecules of the vitreous state are randomly arranged and a unit of structure is not present. The removal of a water molecule of the unit cell of the ice crystal will destroy the fundamental lattice structure by "melting" or fusion. Large amounts of energy are required for the fusion of the unit cell. The corresponding process with vitrified water requires, on the average, much less energy. If the energy of fusion is obtained at the expense of nearby virus particles, dehydration from the crystalline state may disrupt the orderly enzymatic processes of the virus (for example by alterations in high energy phosphate bonds) while dehydration from the vitreous state would be less apt to.

It is also possible that during drying by vacuum sublimation, the removal of water molecules from the crystal lattices results in an instability of the crystals due to the vacancies in the lattices. The replacement of the lost water molecules would be required to restore the initial ordered crystal structures. Bound water of the virus proteins may be the source of water molecules during the final stages of drying. This would result in the precipitation or denaturation of the virus protein with subsequent death.

Nord and his coworkers have shown the existence of transient increases in the activity of frozen solutions of peroxidases, zymases, and tyrosinase under some conditions. They explained these observations by the fact that following exposure to freezing temperatures, lyophilic biocolloid particles in aqueous solutions undergo disaggregation or aggregation according to the concentration of particles present. It seems possible that our observation of an apparent increase in virus particles following some types of lyophilization procedure may likewise be the result of disaggregation.

Effect of Degree of Dehydration.—

Duplicate 0.2 ml. samples of freshly harvested infected allantoic fluid were subjected to the following procedures: rapid cooling to −80° and storage for 48 hours at −40°C.; rapid cooling to −80° and storage for 48 hours at 0°C.; rapid cooling to −80° and vacuum sublimation at 0°C. for 0.5, 1, 2, 4, 8, 12, 24, and 48 hours. The sublimated material was sealed off from the vacuum line at the above intervals and stored at 0°C. until the end of the 48 hour period, at which time the titres of all samples were determined.

After cooling to −80°C. and storage at −40°C. the titre was $10^{-8.6}$. Cooling to −80°C. and storage at 0°C. gave a titre of 10^{-8}. Lyophilization for 0.5, 1, and 2 hours resulted in titres of 10^{-5}, $10^{-6.5}$, and $10^{-8.0}$, respectively; lyophilization for all longer periods did not result in titres significantly lower than that of the control material cooled to −80°C. and stored at −40°C. It may be concluded that lyophilization with our apparatus for 4 hours or longer resulted in sufficient drying to give complete protection against the loss of titre which

resulted on storage of the undried frozen material at 0°C., while lyophilization for even 30 minutes gave partial protection.

Many investigators (38–40) have reported poor stability of biological materials and decreases in the number of viable bacteria and virus particles stored at 18°C. or higher, after partial dehydration which resulted in a water content greater than 0.5 per cent by weight. Flosdorf (39) has suggested that biological activity and the viability of microorganisms may be used as a highly sensitive test of the degree of dehydration.

It should be noted that the high survival rate found in many of our experiments was due, in part, to the presence of protein and other materials in the surrounding allantoic fluid. The protective effect of such materials during the process of lyophilization has been demonstrated by a number of workers (21, 22).

SUMMARY

The infectivity titre of influenza virus–infected allantoic fluid was determined after a variety of procedures involving cyclic slow freezing and thawing, freezing at various rates with subsequent storage at different temperaturess freezing at various rates with subsequent dehydration at various temperatures, and different degrees of dehydration. All these factors were found to influence the survival rate of the virus particles.

Five freeze-thaw cycles resulted in a fall in titre from $10^{-8.6}$ to $10^{-0.8}$, cycles 2, 3, and 4 causing much greater losses than cycles 1 and 5. Rapid cooling to −40°C. or slow cooling to −80 or 190°C. did not cause significant titre loss, but rapid cooling to temperatures above −40° or slow cooling to temperatures above −80°C. caused definite titre loss. Loss of titre on storage occurred only at temperatures above −40°C.

The effect of lyophilization depends both on the preliminary treatment and on the dehydration temperature. Better conservation of titre was obtained after preliminary cooling to −190 or −80°C. than after preliminary cooling to higher temperatures. The most effective sublimation temperatures were 0 and −80°.; the least effective was +20°C. Titre losses in suspensions sublimated at −10, −30, and −60°C. were in general intermediate. No loss in titre occurred after preliminary cooling to −80 or −190°C. and subsequent dehydration at −80 or 0°C. The degree of dehydration definitely affects the survival of virus on storage at 0°C., but sublimation for 4 hours at 0°C. gave complete protection against titre loss on storage at this temperature.

Possible explanations of the observations made are suggested, based on known physiochemical phenomena such as supercooling, vitrification, variations in size and shape of ice crystals with different freezing speeds, differential enzyme inactivation, changes in salt concentration, and changes in energy levels.

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