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Stability of Suspensions of Influenza Virus Dried to Different Contents of Residual Moisture by Sublimation In Vacuo

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After freezing, suspensions of influenza virus were dried by sublimation of water in vacuo to contents of residual moisture of 3.2, 2.1, 1.7, 1, or 0.4%. The stability of the several suspensions was determined by an accelerated storage test. Based on the times predicted for the dried preparations stored at different temperatures to lose 1 log of infectivity titer, the order of stability in relation to residual moistures was as follows: 1.7% > 2.1% > 1% > 3.2% > 0.4%.

The early literature dealing with the end product of drying by sublimation of water in vacuo abounds with such expressions as "bone dry" or "dry as dust." These expressions, interesting in their concept, do not lend themselves to quantitative evaluation. Although there have been speculations and comments about the dangers of overdrying (4, 12, 14, 18, 19), one still finds it stated as axiomatic that moisture content should be reduced to an absolute minimum for maximal stability of freeze-dried biologicals (17). The statement above has been interpreted as a necessity which requires "drying to a residual moisture level of 1% or less" for licensing of dried biologicals. This standard has been adopted also for the preparation of dried biological reference reagents. The absence of experimental data upon which to base an educated judgment concerning the effects of residual moisture on the stability of suspensions of viruses dried by sublimation in vacuo is noteworthy.

The present studies were undertaken to determine the stability of suspensions of influenza virus, as measured by an accelerated storage test (7), after drying by sublimation of water in vacuo to several contents of residual moisture.

MATERIALS AND METHODS

Preparations of purified influenza virus, PR8 strain, suspended in physiological saline plus amounts of calcium lactobionate and serum albumin, human, sufficient to give a final concentration of 1% of each agent, were used in these studies (9). The initial titers of the preparations ranged from $10^{-7.5}$ to $10^{-8.0}$ ID_{50}/0.1 ml as determined in embryonated eggs (6).

To dry the large numbers of samples of virus suspensions necessary for our study, we used a special chamber freeze-drying apparatus, constructed in cooperation with Edwards High Vacuum, International (Manor Royal, Crawley, Sussex, England), possessing capabilities for variations of parameters for drying by sublimation of water in vacuo exceeding those of a manifold dryer (8).

Lyophilization vials (1,450), each containing 1 ml of the virus suspensions, were cooled at approximately 1 C per min to a terminal temperature of −30 C. The vials containing the frozen suspensions of virus were transferred to the precooled (−30 C) shelves of the chamber dryer. Special vials, each containing 12 to 14 ml of suspension, for the determination of residual moisture were treated in a similar manner. After suitable conditions of vacuum had been established in the chamber (10^{-2} to 10^{-4} torr), the temperature of the product was brought slowly to 0 C (12 to 16 hr), and drying by sublimation of water in vacuo continued at that temperature (8).

The first set of samples, consisting of 145 regular vials, for determining infectivity, and two special vials, for determining residual moisture, were removed at the end of 24 hr; the remaining sets were removed at 12-hr intervals thereafter. Sets of dried virus were stored under vacuum at −70 C while residual moistures were determined.

Preliminary studies indicated that none of the components of the virus preparation reacted seriously with the reagents used in the Karl Fischer titration for residual moisture. The Karl Fischer technique (Beckman Automatic Aquameter) was used, therefore, for initial determinations of residual water. Final quantities of water in the dried preparations of virus were determined gravimetrically, drying at +50 C in a vacuum oven to a constant weight.

Attempts to measure residual moisture gravi-

metrically with the samples dried in our regular vials with a 1-ml fill of suspension were difficult and frequently misleading. Because the weights of the dried samples were approximately 10 mg and highly hydrophilic, it was necessary to work in an atmosphere of dry nitrogen; the least absorption of molecules of water, considering the size of the sample, resulted in erroneous and frequently ludicrous values for percentages of residual moisture. To minimize this problem, we used specially constructed vials for our final determinations of water (Fig. 1). The bottoms of these vials were coated with an adhesive plastic containing finely divided particles of aluminum and ground flat to assure good contact between the bottom of the vial and the surface of the shelf of the freeze-drying apparatus.

The data of preliminary studies showed that the rates of drying in both the regular vials and the special vials were comparable, provided that the depths of the suspensions were similar. Our special vials were so calibrated that approximately 12 ml gave the same depth of frozen sample as was contained in a 1-ml fill of our regular vials. The size of the dried samples in the larger vial, approximately 120 mg, gave consistent and reproducible results for residual water even though only ordinary precautions were taken to prevent the ingress of extraneous moisture.

The sets containing approximately 3.0, 2.0, 1.5, 1.0, and 0.5% residual moisture were selected for further study. The experimental design for obtaining the required data and the mathematical analyses necessary to relate stability of dried preparations and their contents of water were based on our recent report on an accelerated storage test for predicting the stability of suspensions of viruses dried by removing water by sublimation in vacuo (7). This test was modified so that samples at 45 C were removed at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 days.

RESULTS

The changes in the infectivity titers of rehydrated suspensions of influenza virus dried to the several contents of residual moisture and exposed to elevated temperatures (28, 36.2, and 45 C) while in the dried state were plotted against time at the elevated temperatures. Based on the "goodness of fit" of the majority of the determined points to a straight line, defined by plotting titer against time on a logarithmic grid and the agreement between the initial concentration as determined by analysis and as given by extrapolation of the plotted curves to zero time, the selection of the equation for a first-order reaction appeared appropriate for determining the rates of degradation (k_1) of dried suspensions. The involved nature of the calculations, (i) for rates of degradation by the method of least squares, and (ii) the statistical test to show that the k_1 values at the three temperatures used for thermal inactivation were significantly different, has led us to program our data for direct computer solution. The k_1 values for the several time-temperature sequences in these studies, for a given content of residual moisture, are given in Table 1. The differences in the k_1 values for a given content of water were statistically significant. The order of k_1 values for a given temperature in relation to residual moisture was as follows: $1.7\% < 2.1\% < 1.0\% < 3.2\% < 0.4\%$. Thus, at each of the three temperatures used for inactivation, the order of declines in titers in relation to residual moisture was as follows: $0.4\% > 3.2\% > 1.0\% > 2.1\% > 1.7\%$.

Based on the observation that a suitable metameter of the potency (the potency itself or its logarithm) declines, at a selected temperature, linearly with time, thermal degradation of dried suspensions of virus should follow the logarithmic form of the Arrhenius relation with respect to absolute temperature (T):

$$\log k_1 = -(\Delta Ha/2.03R)\ (1/T)$$

where k_1 is the specific rate of degradation of the suspension (treated as a first-order reaction), R is the gas constant, and ΔHa is defined as the heat of activation. The equation above indicates also that any value proportional to the specific rate k_1 would permit calculation of the slope of the plot, $\log k_1$ versus $1/T$, and, therefore, the heat of activation. If such a relation is reasonably linear, the degradation rates at lower temperatures can be calculated from the rates obtained at elevated temperatures. A linear relationship was obtained for each set of k_1 values for the suspensions of virus having different contents of water, the plot of k_1 values on a logarithmic scale versus $1/T \times 10^3$ on an arithmetic scale (Fig. 2). This plot

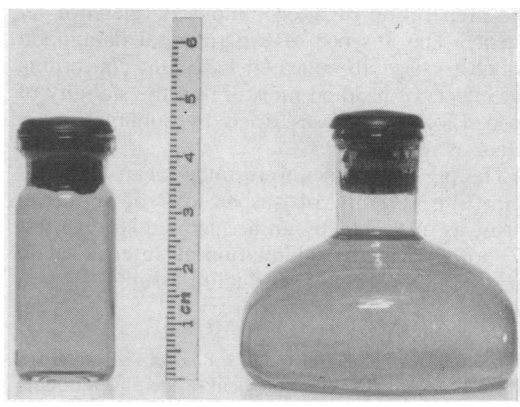

FIG. 1. *Vials for freeze-drying of suspensions of viruses. The standard vial is shown on the left; the special vial for the determination of residual moisture is shown on the right.*

TABLE 1. *Rates of thermal degradation (k_1) for suspensions of influenza virus dried by sublimation of water in vacuo to different contents of residual moisture*

Residual moisture	Temp of inactivation								
	28 C			36.2 C			45 C		
	k_1	SD of k_1	n	k_1	SD of k_1	n	k_1	SD of k_1	n
%									
0.4	0.220	0.0100	14	0.355	0.039	10	0.582	0.092	8
1.0	0.082	0.0094	14	0.177	0.036	10	0.347	0.100	8
1.7	0.039	0.0103	14	0.100	0.040	10	0.230	0.098	8
2.1	0.053	0.0096	14	0.124	0.032	10	0.281	0.103	8
3.2	0.156	0.0106	14	0.278	0.038	10	0.493	0.090	8

permits one to predict the approximate k_1 value for any selected temperature from +50 to −40 C.

Using experimental data, the times required for suspensions dried to the several contents of residual moisture to lose 1 log of titer at each of the elevated temperatures were determined (Table 2). Because the equation relating the rate of degradation (k_1) to the time required to lose 1 log of titer was not known, a straight-line relationship was determined empirically. To encompass times for 1-log loss in titers extending from days to years on a single plot, the log of time was placed on the x axis. Several transformations of the y axis were tested; a straight-line curve was obtained when the logs of the k_1 values, y axis, were plotted against the logs of the times required to lose 1 log of titer, x axis (Fig. 3). The plots obtained allow one to estimate directly the times required for suspensions of influenza virus dried to different residual moistures to lose 1 log of titer for a given value of k_1 and indirectly, therefore, in relation to Fig. 2 the time required to lose 1 log of titer at a selected temperature of storage.

In our previous studies, the reliability of our system was tested by placing vials of dried suspensions of viruses at preselected temperatures and removing the suspensions for assay at the times predicted for a 1-log loss in titer as indicated by our mathematical analysis (7). This approach resulted in long periods of inactivity, prolonged inordinately our test, and at the lower temperatures of storage resulted in an unwelcome degree of uncertainty. To minimize the problems above, we asked of our variables for analysis (k_1 values, temperatures of storage, and times required to lose 1 log of titer) the following question: "At what temperature of storage can we expect to lose 1 log of titer for a preselected time of storage?" The answers to the rhetorical question above, using preselected times of storage of 30 and 60 days, are given in Tables 3 and 4; the predicted infectivity titers and the actual infectivity titers are shown also. These results confirm the reliability of the analysis described as an instrument for predicting the declines in titers of suspensions of influenza virus dried by sublimation of water in vacuo.

The predicted times for suspensions of influenza virus dried by sublimation of water in vacuo to different contents of water to lose 1 log of titer at storage temperatures of +10, 0, −10, and

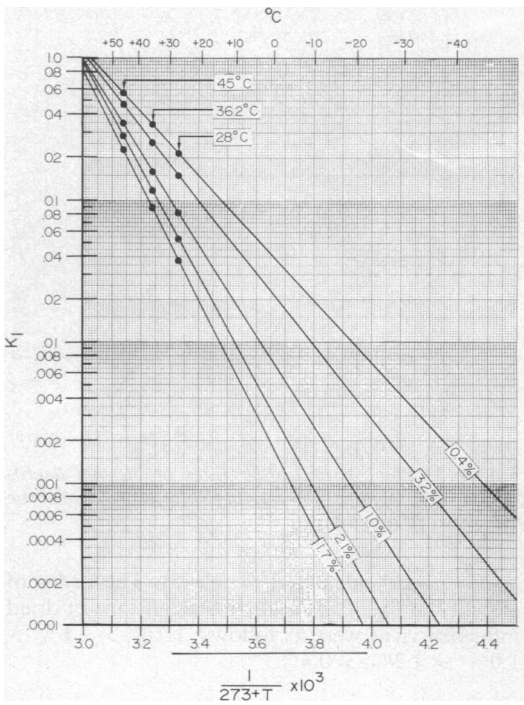

FIG. 2. *Arrhenius plots for suspensions of influenza virus dried by sublimation in vacuo to 3.2, 2.1, 1.7, 1, or 0.4% residual moistures. The curves are the plots of first-order rates of thermal degradation (k_1) against the reciprocals of absolute temperatures ($1/T$).*

TABLE 2. *Experimentally determined times for suspensions of influenza virus, dried to different contents of residual moisture by sublimation in vacuo, to lose 1 log of titer at elevated temperatures*

Residual moisture	Time to lose 1-log titer		
	28 C[a]	36.2 C	45 C
%	*days*	*days*	*days*
0.4	2.0	1.5	1.1
3.2	3.0	2.0	1.3
1.0	6.5	3.4	1.8
2.1	13.0	5.5	2.5
1.7	20.2	8.0	3.5

[a] Temperature of inactivation.

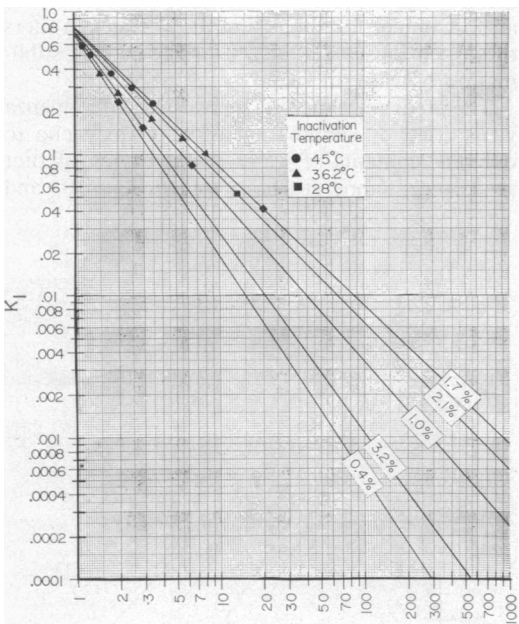

FIG. 3. *Plots of first-order rates of thermal degradation (k_1) against the predicted time required for 1-log loss in titer (days).*

−20 C were calculated (Table 5). The order of stability of the several sets of suspensions of dried influenza virus were as follows: 1.7% > 2.1% > 1.0% > 3.2% > 0.4%.

DISCUSSION

It is generally agreed that molecules of water in biological materials are present in a complex manner. The underlying premise governing the modern concepts of the relation of water to

TABLE 3. *Expected losses in titers and experimental losses in titers for suspensions of influenza virus dried by sublimation of water in vacuo to different contents of residual moisture and stored at temperatures for which 1-log losses in infectivity titers in 30 days were predicted*

Residual moisture	Calculated storage temp for 1-log loss in titer in 30 days	Initial titer (EID_{50})	Titer after storage at calculated temp for 30 days (EID_{50})	
			Expected	Actual
%	C			
0.4	−32	−7.5	−6.5	−6.4
3.2	−15	−7.6	−6.6	−6.6
1.0	+7	−8.0	−7.0	−7.2
2.1	+18	−8.3	−7.3	−7.0
1.7	+25	−8.0	−7.0	−7.2

TABLE 4. *Expected losses in titers and the experimental losses in titers for suspensions of influenza virus dried by sublimation of water in vacuo to different contents of residual moisture and stored at temperatures for which 1-log losses in infectivity titers in 60 days were predicted*

Residual moisture	Calculated storage temp for 1-log loss in titer in 60 days	Initial titer (EID_{50})	Titer after storage at calculated temp for 60 days (EID_{50})	
			Expected	Actual
%	C			
0.4	−43	−7.5	−6.5	−6.6
3.2	−25	−7.6	−6.6	−6.8
1.0	−1.5	−8.0	−7.0	−7.2
2.1	+12	−8.3	−7.3	−7.2
1.7	+18	−8.0	−7.0	−6.8

TABLE 5. *Predicted times for suspensions of influenza virus dried by sublimation of water in vacuo to different contents of residual moisture to lose 1 log of infectivity titer at several temperatures of storage*

Residual moisture	Predicted time to lose 1 log of titer			
	+10 C[a]	0 C	−10 C	−20 C
%	*days*	*days*	*days*	*days*
0.4	4	6	10	16
3.2	7	12	22	40
1.0	24	54	135	480
2.1	68	200	640	>1,000
1.7	145	520	>1,000	>1,000

[a] Temperature of storage.

proteins is that protein molecules having a suitable periodicity of functional groupings can stabilize a lattice of tetrahedrally H-bonded water molecules at the surfaces of proteins (13, 24). It has been suggested also that the "ordered waters" at the surfaces of the proteins serve to stabilize, in part, the native molecular conformation of proteins (2).

The studies of Kendrew and his associates on sperm-whale myoglobin (16), the oxygen-carrying protein of mammalian muscle, indicate the following: (i) protein contains at the most four molecules of water in its interior, (ii) nearly all the polar groups of the amino acids are on the surface of the molecule and, hence, exposed to solvent, (iii) water molecules are attached to all polar groups, giving rise to an ordered water lattice, and (iv) apart from water molecules bound to polar groups, the liquid regions surrounding the protein show no sign of ordering. Although the conclusions above were reached after studies of crystalline protein, the best evidence to date suggests that the crystal structures of proteins and the structures of proteins in solution are closely related (1).

Depending on their position in the structure of protein, molecules of water are probably removed by freeze-drying at different rates and at different times during the process of substracting water by sublimation in vacuo. If one considers the energy requirement for disrupting the lattice structure of the crystalline state of water by "melting" or fusion during sublimation in vacuo in relation to that for nonordered, liquid water, it is highly probable that water in the liquid state would be removed earlier and more easily than water in the ordered, crystalline state. Studies on the activation energies of water neighboring proteins, peptides, and amino acids (16 kcal/mole), and of free water (4 kcal/mole), support the above concept (5). Thus, it would appear that during the removal of water from suspensions of viruses by sublimation in vacuo, free water will be removed before ordered water attached to the protein of the surface of the virus will sublimate.

Denaturation has been defined as a process (or a sequence of processes) in which the spatial arrangements of the polypeptide chains within the molecule are changed from that typical of the native protein to a more disordered arrangement (15). It is important to realize that the results of studies of denaturation are frequently functions of the types of measurements that are made and that loss of biological function is the most sensitive test, even though physical measurements reveal only slight alterations in structure or no changes at all. The peptide chains of the closely folded native protein cannot unfold unless water flows into the spaces between the chains; dry proteins are much more resistant to heat denaturation than proteins in solution (11). Thus, the free water at the surfaces of the proteins of the viral membranes that have been underdried, 3.2% residual moisture, will be available for transconformation of the protein. The rate of change of protein configuration will be dependent, in part, on the rate of diffusion of water molecules through ice crystals and will be a function of the temperature of storage of the dried preparation, i.e., the lower the temperature the slower the rate of diffusion (21).

Studies of the oxidation of the protein fraction of beef muscle dried by sublimation of water in vacuo show that these preparations oxidize at a rate sufficient to account for 50% or more of the oxygen absorption of freeze-dried beef (20, 23). The oxygen absorption of lactalbumin, gelatin, and purified or crystalline proteins, prepared in a similar manner to the above, suggests that oxygen lability may be a general property of dried proteins. These results are consistent with the observations that low moisture levels (overdrying) are frequently conducive to oxidation (22). The reduced water-combining capacity of proteins after freeze-drying supports further the hypothesis of oxidation of hydrophilic sites after the removal of structured water by sublimation in vacuo (3, 10).

Overdrying, 0.4% residual moisture, by removing the "bound water" of the protein coat of the virus particle may bring about the following: (i) removal of the stabilizing properties of the water lattice and a consequent modification of protein confirmation, and (ii) direct exposure of the hydrophilic sites at the protein surface to the gasses present in the atmosphere surrounding the dried protein. Even when dried virus preparations were sealed under vacuum (10^{-4} torr) in 5-ml vials, the atmosphere surrounding the dried preparations would contain 3.5×10^{12} molecules of O_2 (at a 1:1 ratio of hydrophilic sites to molecules of oxygen), an amount sufficient to modify many of the protein molecules present. Our data indicated also that the rates of oxidation were a function of the temperatures and times of storage.

The small losses in infectivity titers of the overdried preparations during and immediately after freeze-drying, in contrast to the losses in activities after storage at low temperatures, suggest that oxidation (long-range effect) plays a greater role in the degradation of dried viruses than do changes in protein conformation (short-range effect). Thus, the inactivation of overdried suspensions of viruses results primarily from the

inability of the protein coat of these preparations to rehydrate.

The foregoing discussion postulates two mechanisms for the degradation of viruses dried by sublimation of water in vacuo: (i) changes in protein configuration associated with underdrying, 3.2% residual moisture, and (ii) saturation or blocking of hydrophilic sites of proteins, by oxidation, associated with overdrying, 0.4% residual moisture. As these mechanisms are based on the amounts and kinds of water remaining after freeze-drying, one could reasonably predict increased stability of suspensions dried to contents of residual water intermediate to the above, an expectation consistent with the increased stability of suspensions of virus dried to a residual moisture content of 1, 1.7, or 2.1%. Further, maximal stability would be predicted for that suspension dried to a content of residual water where most, if not all, of the "free water" was removed and sufficient amounts of "ordered water" remained to stabilize protein structures and to protect hydrophilic sites of the proteins from oxidation. Our data indicate that maximal stability of suspensions of influenza virus dried by sublimation of water in vacuo occurred at a residual moisture content of approximately 1.7%.

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