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Stabilities of Suspensions of Influenza Virus Dried by Sublimation of Ice In Vacuo to Different Contents of Residual Moisture and Sealed Under Different Gases

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Suspensions of influenza virus were dried by sublimation of ice in vacuo to contents of residual moisture of 2.8, 1.6, or 0.6%. After drying, the preparations were sealed under helium, argon, nitrogen, or a mixture of oxygen and nitrogen (30% O_2, 70% N_2). Stabilities of the several preparations were determined by an accelerated storage test. Based on the times predicted for the dried preparations stored at preselected temperatures to lose 1 log of infectivity titer, the order of stabilities in relation to residual moisture, gas was as follows: 1.6%, He > 0.6%, He > 2.8%, He > 1.6%, Ar > 2.8%, N_2 > 2.8%, Ar > 1.6%, N_2 > 2.8%, O_2 > 0.6%, Ar > 0.6%, N_2 > 1.6%, O_2 > 0.6%, O_2. The stability of the preparation sealed under helium and dried to the content of residual moisture found best for this gas (1.6%) resulted in an increased stability of the order of years as compared to the other preparations tested.

Recent studies of the effects of dehydration on diverse biological materials have shown an optimum content of residual moisture to be necessary for maximum stability (1, 5, 8). These results are opposed to the generally accepted and older view that product stability increases as the content of residual moisture decreases. Those studies indicated that it was not the absolute water content that was decisive but rather the way in which water was bound and how it was available.

Dried preparations used for investigating the effects of contents of residual moisture on stability have been sealed in a vacuum (atmosphere under reduced pressure) or under dried nitrogen. The effects of sealing under gases possessing different chemical, physical, and thermodynamic properties have been studied in a limited manner only (6). The present studies were undertaken to determine the stabilities of suspensions of influenza virus dried by sublimation of ice in vacuo to different contents of residual moisture and sealed under helium, argon, nitrogen, or a mixture of oxygen and nitrogen (30% O_2, 70% N_2).

MATERIALS AND METHODS

Preparations of purified influenza virus (2) 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 (7). Lyophilization vials, 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 suspension of virus were transferred to the precooled (−30 C) shelf of our chamber dryer. Special vials, each containing 12 to 14 ml of suspension, for the determination of residual moisture were treated in a similar manner (5). 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 ice in vacuo continued at that temperature (4).

The first set of samples was sealed internally under vacuum and removed at the end of 20 hr, a period of lyophilization expected to result in a content of residual moisture of approximately 3%. The second set of samples was removed at the end of 28 hr of lyophilization (an expected content of residual moisture of 1.5%). The third set was removed at the end of 48 hr (an expected content of residual moisture of 0.5%). By using the special vials, the content of residual moisture in the three sets of samples were determined gravimetrically (5). The results obtained were as follows: 20 hr of drying, 2.8%; 28 hr of drying, 1.6%; 48 hr of drying, 0.6%. After removal from the freeze-drying chamber, the sealed vials containing the dry suspensions of virus were stored at −70 C while awaiting further manipulation.

Subsets of the three initial sets of samples were re-

moved from the deep-freeze, and the stopples were removed carefully in an atmosphere of dry nitrogen. New stopples were inserted, and the vials were returned to the chamber freeze-dryer. The chamber was evacuated to 10^{-3} torr. Vacuum was broken with one of the several gases being tested. Three cycles of evacuation and refilling with a given gas were carried out. At the end of the third cycle, bladders of the internal sealing mechanism were activated with dry nitrogen under pressure, and the dried suspensions were sealed under gas at a pressure of 1 atm (5). The rubber stopples used in these studies were pretreated by heating to 50 C in a vacuum of 10^{-4} torr for 24 hr to remove intrapped water, gases, and volatile agents. The rubber stopples were fixed in place with aluminum seals, and the vials of dried virus under the several gases were returned to the deep-freeze and kept at −70 C until further testing was carried out.

After all subsets had been prepared, they were divided into groups and placed in incubators at 28, 36.2, and 45 C. These incubators had a temperature variation of ±0.2 C. Samples were removed at various elapsed times and stored at −70 C until titers were determined (2). The experimental design for obtaining the required data and the mathematical analyses necessary to relate stabilities of preparations, dried to different contents of residual moisture and sealed under different gases, were based on our report on an accelerated storage test for predicting the times required for dried suspensions of viruses to lose 1 log of titer when stored at preselected temperatures (3).

RESULTS

The rates of thermal degradation (k_1) at 28, 36.2, or 45 C for suspensions of influenza virus dried by sublimation of ice in vacuo to contents of residual moisture of 2.8, 1.6, or 0.6% and sealed under helium, argon, nitrogen, or oxygen (30% O_2, 70% N_2) are shown in Table 1. The rates obtained allow for many interesting and important comparisons, for example, that (i) thermal degradation of dried suspensions sealed under oxygen was less in underdried samples (2.8% residual moisture) than in the sets of other samples sealed under this gas (1.6 or 0.6% residual moisture) or that (ii) the smallest values for thermal degradation were associated with samples sealed under helium. However, the development of other relationships can be left to the reader, allowing this investigator to devote time and space to the main thrust of these studies, which is the predicted stabilities, in real time, of dried suspensions.

Previous studies have shown that the thermal degradation of dried suspensions of influenza virus is like any other time- and temperature-dependent chemical reaction and follows, therefore, the logarithmic form of the Arrhenius relation with respect to absolute temperature (T; reference 3). If, for a given set of samples, the plot of the logs of k_1 values versus $1/T$ is reasonably linear, rates of degradation at lower temperatures can be calculated from the rates obtained at elevated temperatures. The conditions above were found to hold for the present studies. Further, the steeper the slope of the plot obtained for a given set, the smaller is the value of k_1 for a given temperature and therefore the more stable the preparation. The older of stabilities of the several sets

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

Gas	Residual moisture (%)	Rates of k_1 at		
		28 C[a]	36.2 C	45 C
Helium	2.8	0.077	0.129	0.248
Argon	2.8	0.100	0.162	0.285
Nitrogen	2.8	0.094	0.150	0.273
Oxygen	2.8	0.135	0.203	0.344
Helium	1.6	0.042	0.074	0.160
Argon	1.6	0.078	0.118	0.222
Nitrogen	1.6	0.109	0.163	0.295
Oxygen	1.6	0.188	0.260	0.424
Helium	0.6	0.084	0.148	0.295
Argon	0.6	0.175	0.267	0.444
Nitrogen	0.6	0.192	0.290	0.473
Oxygen	0.6	0.271	0.360	0.525

[a] Temperature of inactivation.

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

Gas	Residual moisture (%)	Time to lose 1 log of titer at		
		28 C[a] (days)	36.2 C (days)	45 C (days)
Helium	2.8	11.5	8.0	5.0
Argon	2.8	5.5	4.0	3.0
Nitrogen	2.8	8.0	6.0	5.0
Oxygen	2.8	3.0	2.50	1.75
Helium	1.6	40.0	22.0	11.0
Argon	1.6	9.5	7.0	4.5
Nitrogen	1.6	5.0	3.5	2.5
Oxygen	1.6	2.50	2.0	1.50
Helium	0.6	8.0	5.5	3.5
Argon	0.6	3.75	3.0	1.75
Nitrogen	0.6	3.0	2.5	1.50
Oxygen	0.6	1.75	1.50	1.25

[a] Temperature of inactivation.

(content of residual moisture, gas) was as follows: 1.6%, He > 0.6%, He > 2.8%, He > 1.6%, Ar > 2.8%, N_2 > 2.8%, Ar > 1.6%, N_2 > 2.8%, O_2 > 0.6%, Ar > 0.6%, N_2 > 1.6%, O_2 > 0.6%, O_2.

To prepare the plots relating the values of k_1 to the times required for the several sets of samples to lose 1 log of titer at selected temperatures of storage, the experimentally determined times for the several sets to lose 1 log of titer at elevated temperatures were calculated (Table 2).

By using the graphs "k_1 versus $1/T$" and "k_1 versus time to lose 1 log of titer" (3, 5, 6) we were able to estimate (or predict) the times required for the several sets to lose 1 log of titer when stored at 20, 10, 0, −10, or −20 C (Table 3).

At the three contents of residual moisture, the times predicted for dried preparations to lose 1 log of titer for all projected temperatures of storage were highest in those samples sealed under helium. Among the preparations sealed under helium, samples dried to a content of residual moisture of 1.6% were the most stable at each projected temperature of storage. Those dried to a content of residual moisture of 0.6% were the least stable, and those dried to 2.8% residual moisture were intermediate. The differences in the values for the predicted times to lose 1 log of titer for a given projected temperature of storage of preparations dried to contents of residual moisture of 2.8 and 0.6% and sealed under helium were in the order of days, varying from 6 days at 20 C to 50 days at the −20 C. The differences for the above between samples dried to a content of residual moisture of 1.6% and those dried to contents of residual moisture of 2.8 or 0.6% were several orders of magnitude greater, varying from days at the highest temperatures of storage, 20, 10, or 0 C, to years at the lower temperatures of storage, −10 or −20 C.

At the three contents of residual moisture, the times predicted for dried preparations to lose 1 log of titer for a given projected temperature of storage were lowest in those samples sealed under 30% oxygen. For a given projected temperature of storage, samples dried to a content of residual moisture of 2.8% were most stable. Those dried to a content of residual moisture of 0.6% were least stable, and those dried to 1.6% residual moisture were intermediate. Predicted times for losses of 1 log of titer ranged from a low of 2 days, for preparations dried to a content of residual moisture of 0.6% with a projected temperature of storage of 20 C, to 21 days, for preparations dried to a content of residual moisture of 2.8% with a projected temperature of storage of −20 C.

The value for the predicted times to lose 1 log of titer for a given projected temperature of storage in preparations dried to contents of residual moisture of 2.8 or 0.6% and sealed under argon was of the same order of magnitude. A significant increase in stabilities was found for samples dried to 1.6% residual moisture and sealed under argon.

Approximately the same orders of stability were found in preparations dried to contents of residual moisture of 1.6 or 0.6% and sealed under nitrogen. Preparations dried to a content of residual moisture of 2.8% and sealed under nitrogen, however, showed a twofold increase in stabilities for a given projected temperature of storage.

For a given projected temperature of storage, preparations dried to a content of residual moisture of 2.8% and sealed under nitrogen took ap-

TABLE 3. *Predicted times for suspensions of influenza virus dried to different contents of residual moisture and sealed under different gases to lose 1 log of infectivity titer at several storage temperatures*

Gas	Residual moisture (%)	Predicted time to lose 1 log of titer at				
		20 C (days)	10 C (days)	0 C (days)	−10 C (days)	−20 C (days)
Helium	2.8	18	27	56	105	220
Argon	2.8	7.5	11	17	26	43
Nitrogen	2.8	12	20	33	57	108
Oxygen	2.8	4	6	9	14	21
Helium	1.6	70	140	330	800	2,050
Argon	1.6	14	25	43	76	150
Nitrogen	1.6	7	10	17	27	50
Oxygen	1.6	3.5	4.5	6.5	9.5	15
Helium	0.6	12	21	42	80	170
Argon	0.6	5	7.5	13	23	40
Nitrogen	0.6	4.5	7.0	11	20	35
Oxygen	0.6	2.0	2.75	3.5	4.5	6.0

proximately twice as long to lose 1 log of titer than did similar preparations sealed under argon. On the other hand, preparations dried to a content of residual moisture of 1.6% and sealed under argon took approximately three times as long to lose 1 log of titer for a given projected temperature of storage than did similar preparations sealed under nitrogen. Differences in predicted times to lose 1 log of titer for a given projected temperature of storage were not significant in preparations dried to a content of residual moisture of 0.6% and sealed under argon or nitrogen.

DISCUSSION

We have previously shown that both underdrying (3.2% residual moisture) and overdrying (0.6% residual moisture) were conditions adversely affecting the stabilities of suspensions of influenza virus dried by sublimation of ice in vacuo and sealed in a vacuum (essentially atmosphere at reduced pressure). At contents of residual moisture intermediate to the extremes above, dried preparations showed increasing stabilities with maximum stability achieved in preparations dried to a content of residual moisture of 1.7%. The interpretation of the results obtained was based on the amounts and kinds of water present after freeze-drying in relation to the protein coat of the virus particle: "structural water" had water molecules bound to carboxy and amino groups of proteins; "random water" had water molecules present on but not chemically or physically adsorbed to the surface of proteins. It was postulated that overdrying by removing both random and structural waters would lead to denaturation of the protein present by exposing the hydrophilic sites at the protein surface to oxidation. Under these circumstances, we would expect the water barrier present in underdried preparations to protect the protein surface of virus particles from the effects of oxygen, and the greater the barrier, the greater would be the stabilities of preparations sealed under this gas. The order of stabilities in relation to contents of residual moisture for preparations sealed under oxygen observed in the present studies, 2.8% > 1.6% > 0.6%, support the hypothesis above. A similar order of stabilities in regard to contents of residual moisture was observed in preparations sealed under nitrogen. This finding raises the possibility that nitrogen, like oxygen, may combine with the exposed sites on the surfaces of proteins leading to denaturation or, by blocking hydrophilic sites, may prevent rehydration.

The role of residual moisture in relation to the stability of dried preparations of biological materials has been recognized and studied (5); the part played by sealing under different gases has received less attention (6). In general, it has been the practice to dry to contents of residual moisture of 1% or less when sealing dried samples in a vacuum (a mixture of gases at reduced pressures) or under nitrogen or argon. Under these conditions, dried products with increased stabilities, when compared to the frozen products stored at low temperatures (−20 to −70 C), were obtained. Although limited number of gases were used in the present studies and the suspensions of influenza virus were dried to only three different contents of residual moisture, the data indicate that the content of residual moisture for maximum stability varies with the gas under which the dried product is sealed. The order of stabilities of preparations dried to different contents of residual moisture and sealed under helium or argon was 1.6% > 2.8% > 0.6%; the order for preparations sealed under nitrogen or oxygen was 2.8% > 1.6% > 0.6%. In all cases, overdried preparations were the most labile. These data also indicate that flushing with and sealing under helium improves the stabilities of dried preparations. Finally, it is to be noted that the stabilities of preparations sealed under helium and dried to the content of residual moisture found best for this gas, approximately 1.6%, results in increased stabilities of the order of years.

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