APPLIED MICROBIOLOGY, June 1969, p. 830-835

Vol. 17, No. 6
Printed in U.S.A.

Stabilities of Dried Suspensions of Influenza Virus Sealed in a Vacuum or Under Different Gases

DONALD GREIFF AND WILTON A. RIGHTSEL

Department of Pathology, Marquette School of Medicine, Inc., Milwaukee, Wisconsin 53233, and Department of Pathology, Baptist Memorial Hospital and Department of Microbiology, University of Tennessee School of Basic Medical Sciences, Memphis, Tennessee 38103

Received for publication 5 March 1969

Suspensions of purified influenza virus, dried to a 1.4% content of residual moisture by sublimation of ice in vacuo, were sealed in a vacuum or under different gases of high purity. The stabilities of the several preparations were 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 stabilities in relation to sealing in vacuum or under different gases was as follows: helium > hydrogen > vacuum > argon > nitrogen > oxygen > carbon dioxide.

Modern techniques of drying by sublimation of ice in vacuo are today replacing older methods of storage at extremely low temperatures as the preferred method for preserving the active components of biological materials. This is especially true in regard to viral vaccines and diagnostic reference reagents. The morphological and biochemical differences of the many agents or reagents being investigated has made it necessary to carry out studies to define the optimal conditions for freeze-drying (7, 21). The objective of the present study was to compare the relative stabilities of dried preparations of influenza virus stored under the generally approved methods of vacuum or nitrogen with storage under other gases.

In recent years, interest in noncatalytic, heterogeneous gas-solid reactions has increased primarily because of improved physical and mathematical procedures for analyzing the reaction systems (11, 12, 15). Two factors must be taken into consideration: (i) the rate of diffusion of the gas into the solid and (ii) the chemical reaction between gas and solid. The first factor depends on the molecular mass of the gas, the pore sizes of the solid, and the nature of the dried matrix; the second depends on the chemical nature of the gas and the reactive sites present on or in the solid. In the majority of biological materials dried by sublimation of ice in vacuo, pore size is such that gases penetrate rapidly throughout the solid (11, 12) and chemical reactions depend on the amounts of water removed, the reactive sites of the biological material exposed by removal of water, and the specific activities of the gas (8).

The method of using elevated temperatures to accelerate degradation and thus mathematically to predict the stabilities of dried suspensions of viruses (6, 8) was used in the present study to determine the effects of storing influenza virus, dried by sublimation of ice in vacuo, under different gases. The gases selected were those which would give insight into gas-solid reactions or were those used commonly for preparing viral vaccines or that were contaminants of such gases. The gases studied were the following: helium and hydrogen gases of small molecular mass; oxygen and carbon dioxide gases of moderate molecular mass but of high chemical reactivity; argon and nitrogen gases of moderate molecular mass but of low chemical reactivity or inert.

MATERIALS AND METHODS

Preparations of purified influenza virus, PR8 strain, suspended in physiological saline plus 1% calcium lactobionate (w/v) and 1% human serum albumin (v/v), were used in these studies (10). The initial infectivity titer of the preparation, as determined in embryonated eggs, was $10^{-7.8}$ ID_{50}/0.1 ml (5). Lyophilization vials (6 ml), each containing 1 ml of the virus suspension, 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 shelves of a chamber dryer precooled to −30 C. Special vials, each containing 12 to 14 ml of suspension, for the determination of residual moisture (8) 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 preparation was brought slowly to 0 C over a 16-hr

period and drying by sublimation of ice in vacuo continued at that temperature (7); total drying time was 48 hr. At the end of the drying period, the vials were sealed with special rubber stopples under a vacuum of 10^{-4} torr within the vacuum chamber, removed, and stored at −70 C for further study.

By use of the dried preparations of virus in the special vials, residual moisture was determined gravimetrically (8) and was found to be 1.4%.

Three-hundred sealed ampoules were removed from the deep-freeze, and the stopples were removed carefully in an atmosphere of dry nitrogen. New vacuum 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, the 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. 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 entrapped water, gases, and volatile agents. The rubber stopples were fixed in place with aluminum seals, and the vials of dried virus were returned to the deep-freeze and kept at −70 C until further testing was carried out.

The gases and the mixture of gases used in these studies (air, nitrogen, carbon dioxide, a mixture of 30% oxygen–70% nitrogen, argon, hydrogen, and helium) were of high purity (Linde) and were reported to contain 3 to 5 ppm of water vapor. Taking into consideration (i) potentially deleterious effects of elevated amounts of water vapor on the stabilities of dried suspensions, (ii) the relatively small numbers of water molecules required to raise the water content of the dried samples to dangerous levels, and (iii) the lack of care which sometimes accompanies the storing or filling of gas cylinders, we took precautions to reduce the dew point of the gases used to −75 C, 1.5 ppm of water vapor, before admitting the gases into the evacuated chamber of the freeze-dryer. This was accomplished by passing the gases through columns of a molecular sieve (Linde, 4A) cooled to −76 C.

The experimental design, the kinds of data needed, and the mathematic analyses required for the present investigations were similar to those of our recent studies on the relationships between the stabilities of dried preparations of influenza virus and their contents of residual moisture (8).

RESULTS

The changes in the infectivity titers of rehydrated suspensions of influenza virus dried to a 1.4% content of residual moisture, sealed under different gases or under vacuum, and exposed to elevated temperatures (28, 36.2, or 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 of the initial concentration, as determined by experiment 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 sealed under different gases (6). The involved nature of the calculations for rates of degradation by the method of least squares has led us to program our data for direct computer solution. The k_1 values for the several time-temperature sequences for a given gas are shown in Table 1. The order of k_1 values for a given temperature, in relation to the gas used, was as follows: He < H_2 < vacuum < Ar < N_2 < O_2 (as O_2–N_2) < CO_2. Thus, at each of the three temperatures used for inactivation, the order of decline in titers in relation to the gas used was as follows: CO_2 > O_2 (as O_2–N_2) > N_2 > Ar > vacuum > H_2 > He.

Based on the observation that infectivity titers or their logarithms decline, at a selected temperature, linearly with time, thermal degradation of dried suspensions of virus should follow the logarithm form of the Arrhenius equation with respect to absolute temperature. If the plot of log k_1 versus $1/T$ is reasonably linear, degradation rates at lower temperatures can be calculated from the rates obtained at elevated temperatures; the plot of k_1 values on a logarithm scale versus $1/T \times 10^3$ on an arithmetic scale is shown in Fig. 1. This plot permits one to predict the approximate k_1 value for any selected temperature from 50 to −40 C.

By use of the experimental data, the times required for dried preparations of influenza virus, sealed under different gases or vacuum, to lose 1 log of titer at each of the elevated temperatures (28, 36.2, or 45 C) were determined (Table 2). Because the equation relating the rates of degradation, k_1 values, to the times required for 1-log

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

Gas	Rates of k_1 at		
	28 C[a]	36.2 C	45 C
Helium	0.051	0.077	0.150
Hydrogen	0.058	0.095	0.172
Vacuum	0.070	0.117	0.185
Argon	0.082	0.132	0.225
Nitrogen	0.102	0.150	0.245
Oxygen (30%)	0.134	0.183	0.260
Carbon dioxide	0.191	0.248	0.351

[a] Temperature of inactivation.

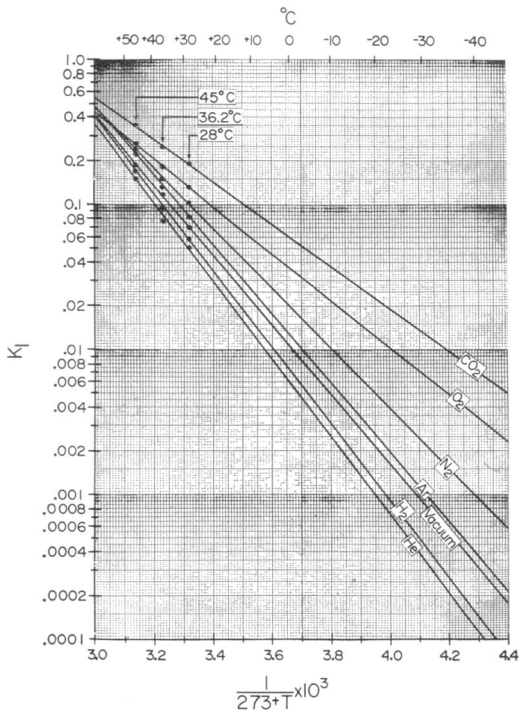

FIG. 1. *Arrhenius plots for suspensions of influenza virus dried by sublimation of ice in vacuo and sealed under different gases. The curves are the plots of first-order rates of thermal degradation (k_1) against the reciprocals of absolute temperatures ($1/T$).*

TABLE 2. *Experimentally determined times for suspensions of influenza virus to lose 1 log of titer at elevated temperatures*[a]

Gas	Time to lose 1 log of titer at		
	28 C[b]	36.2 C	45 C
	days	*days*	*days*
Helium	34.0	20.0	9.0
Hydrogen	24.0	12.5	6.0
Vacuum	12.5	8.5	5.0
Argon	8.5	6.0	3.5
Nitrogen	6.5	4.75	3.0
Oxygen (30%)	3.5	3.0	2.5
Carbon dioxide	2.0	1.75	1.5

[a] Suspensions were dried by sublimation of ice in vacuo and sealed under different gases.
[b] Temperature of inactivation.

losses in 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; as with our previous studies on influenza virus (8), a straight-line curve was obtained when the logs of the k_1 values were plotted on the y axis against the logs of the times required to lose 1 log of titer (Fig. 2). The plots obtained allow one to estimate directly the times required for suspensions of influenza virus, dried to a content of residual moisture of 1.4% and sealed under different gases or under vacuum, to lose 1 log of titer for a given value of k_1 and therefore to estimate indirectly, in relation to Fig. 1, the time required to lose 1 log of titer at a selected temperature of storage.

The applicability of this system to dried suspensions of influenza virus was shown previously (8). Confirmation was obtained in the present study by exposing the several preparations to those temperatures at which it was predicted that a 1-log loss in titer would occur in 30 days (Table 3).

The predicted times for suspensions of influenza virus, dried by sublimation of ice in vacuo to a residual moisture content of 1.4% and sealed under different gases or under vacuum, to lose 1 log of titer at storage temperatures of 20, 10, 0, −10, and −20 C were calculated (Table 4). The order of stabilities of the several sets of suspensions of dried influenza virus was as follows:

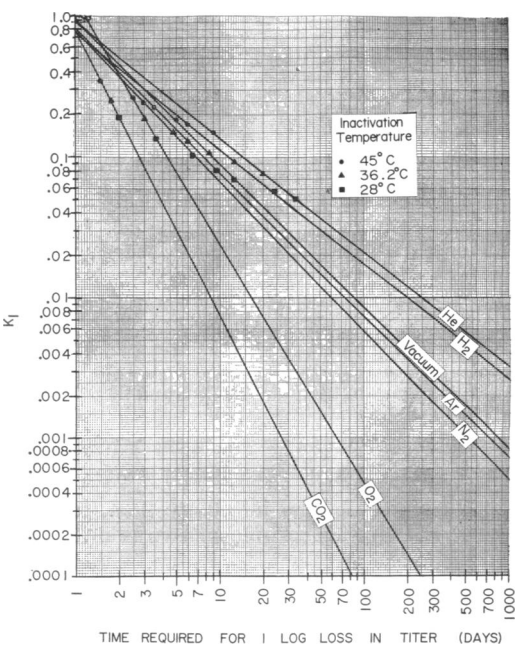

FIG. 2. *Plots of first-order rates of thermal degradation (k_1) against the predicted time required for a 1-log loss in titer (days).*

TABLE 3. *Losses in titers for suspensions of influenza virus*[a]

Gas	Calculated storage temp for 1-log loss in titer in 30 days	Titer after storage for 30 days (EID_{50})
	C	
Helium	30	−6.4[b]
Hydrogen	25	−6.6
Vacuum	15	−7.0
Argon	10	−7.0
Nitrogen	0	−6.8
Oxygen (30%)	−40	−6.4
Carbon dioxide	−70	−6.3

[a] Suspensions were dried by sublimation of ice in vacuo, stored under different gases and under vacuum, and maintained at temperatures for which 1-log losses in infectivity titers in 30 days were predicted. The initial titer (EID_{50}) was −7.8.

[b] A titer of −6.8 was expected for all of the gases.

$He > H_2 >$ vacuum $> Ar > N_2 > O_2$ (as O_2–N_2) $> CO_2$.

DISCUSSION

General considerations. To maintain activity after freezing and thawing or freeze-drying and rehydration, the structural and functional organization of viruses must be considered with regard to the following facts: (i) the essential infective component is a high-molecular-weight nucleic acid; and (ii) a protective coat, predominately protein, is present which serves to transmit the infective core in a functionally intact state through space and time to a susceptible host. The surfaces of virus particles, serving as limiting boundaries, bear the first brunt of assault by phasic changes during freezing and the initial losses of water during freeze-drying. Thus, the kinds of changes in the proteins of the surfaces of virus particles during freezing, freeze-drying, and long term storage at low temperatures would be expected to influence, in part, the activities of rehydrated particles.

Almost all properties of proteins are modified, often to a very large extent, when denaturation takes place. In spite of their multiplicity, all of the phenomena related to the denaturation processes are consequences of fundamental changes in structure. Denaturation is always accompanied by changes in the free energy which are involved in the destruction of the secondary bond structure and modification in contacts with the surrounding medium. With the k_1 values for the several time-temperature relationships required for predicting the stabilities of suspensions of viruses, we have subjected our data to thermodynamic analyses, based on Eyring's theory of absolute reaction rates, to determine changes in (i) the Gibbs' free energy function, (ii) the heat of activation, and (iii) the entropy of activation for the degradation process (4, 9). The values obtained indicated that we were dealing with three sets of data: (i) a set consisting of preparations sealed under helium or hydrogen; (ii) a set consisting of preparations sealed under argon, nitrogen, or vacuum; and (iii) a set consisting of preparations sealed under oxygen or carbon dioxide.

Based on our recent studies on the stabilities of suspensions of influenza virus dried to different contents of residual moisture by sublimation of ice in vacuo, virus suspensions in the present study were slightly overdried (8). Overdrying, by removing the random water and some of the structured water of the protein coat of the virus particle, may bring about the following: (i) modification of protein configuration and (ii) direct exposure of the hydrophilic sites at the protein surface to the different gases.

TABLE 4. *Predicted times for suspensions of influenza virus to lose 1 log of infectivity titer at several storage temperatures*[a]

Gas	Predicted time to lose 1 log of titer at				
	20 C	10 C	0 C	−10 C	−20 C
	days	*days*	*days*	*days*	*days*
Helium	80	180	460	>1,000	>1,000
Hydrogen	47	130	290	800	>1,000
Vacuum	21	39	82	170	390
Argon	16	29	60	125	280
Nitrogen	11	18	31	59	115
Oxygen	4.5	6	8	13	15
Carbon dioxide	2.3	2.9	3.5	4.5	5.8

[a] Suspensions were dried by sublimation of ice in vacuo and stored under different gases.

One may criticize many studies of the effects of sealing under dried gases on the basis that the methods used did not dry the gases adequately. In those reports in which the methods of drying were not given, one can only assume that the word of the gas distributor as to the water content was accepted or that the importance of the water content was not appreciated. The results obtained, therefore, cannot be accepted with complete certainty.

Vacuum. Although there is a tendency to regard the phrase "sealed in a vacuum" as synonymous with "nothingness," in reality this phrase indicates only that the gases found in air are present in reduced amounts. The major gas molecules present include: oxygen, nitrogen, argon, carbon dioxide, helium, hydrogen, and water. The amounts of the above in containers evacuated to 10^{-4} torr ranged from 0.5 to 20 ppm. The contaminating molecules present in the "high purity" gases (Linde) and in the mixture of gases used in the present study were similar in kinds and amounts to the above. Thus, the titers of preparations of dried virus sealed under vacuum served as "blank values" for changes in activities.

Oxygen. Several studies have shown that, in general, dried suspensions of bacteria or rickettsiae stored under vacuum are more stable than similar suspensions stored under dried air or dried oxygen (1, 13, 16–20). Inactivation of virus particles in the slightly overdried preparations used in our study may result from oxidation of exposed hydrophilic sites at the surfaces of proteins (8). It has also been shown that irreversible denaturation of protein is distinctly more rapid in the presence than in the absence of oxygen (14). Thus, the marked declines in the titers of preparations of dried influenza virus stored under 30% oxygen may be the result of oxidation of all of the exposed hydrophilic sites at the surfaces of viral protein (chemical) and an accelerated, irreversible denaturation of viral protein (physical).

Carbon dioxide. The myxoviruses are characterized as being acid-labile. The inactivation of suspensions of influenza virus stored in a chest with dry ice occurs on thawing when the CO_2 dissolved in the frozen preparation unites with the water of melting ice to form carbonic acid. To determine whether the inactivation of virus stored under carbon dioxide was caused by the formation of carbonic acid upon rehydration, the following studies were undertaken: (i) rehydrating dried suspensions with a phosphate buffer at *p*H 7.5; (ii) flushing sealed vials with dry nitrogen or with dry helium before rehydration with water; (iii) evacuating sealed vials, 10^{-4} torr, before rehydration with water. In none of these studies were the k_1 values significantly different from those of preparations sealed under carbon dioxide and rehydrated with water in the presence of carbon dioxide. Therefore, it appears that deleterious modifications of the virus particle during storage may result from the combination of carbon dioxide with the structured water of proteins or with other chemical components.

Nitrogen and argon. For many years, biological materials dried by sublimation of ice in vacuo have been sealed under nitrogen gas. It has been accepted as an article of faith that nitrogen gas does not react with materials dried in this manner. In those few studies in which data were obtained showing storage under nitrogen to be deleterious (2, 17, 19), we were uncertain as to whether the effects observed could be assigned primarily to the nitrogen or to the molecules of water present in the gas. The data of our studies indicated that the decreased k_1 values for preparations of virus stored under nitrogen which had been dried to a dew point of -75 C may be real as compared to those values for storage under vacuum. Thus, under the experimental conditions used in our studies, nitrogen may have a deleterious effect on dried virus.

Our studies also showed that the infectivities of dried viral preparations sealed under dry argon gas were not significantly different from those sealed under vacuum. A study by Coady indicated similar results (M. G. Coady, U.S. Patent Office no. 3, 143, 471).

Helium and hydrogen. The molecular masses of helium (He) and of hydrogen (H_2) are lower by several orders of magnitude than those of the other gases used in our study; their average velocities (centimeters per second), on the other hand, are higher by several orders of magnitude (3). Based on these properties, the molecules of helium and hydrogen would migrate rapidly and with minimal impedance throughout the dried viral preparation. At the end of the flushing process, when the pressure in the total system equals 1 atm, the number of hydrogen or helium molecules in dried preparations would exceed those of oxygen, nitrogen, carbon dioxide, and water remaining after drying virus suspensions by sublimation in vacuo. It would be predicted, on the basis of the kinetic theory of gases, that the molecules of hydrogen or helium could undergo elastic collisions with the molecules of oxygen, nitrogen, carbon dioxide, and water and in the process give up energy to these molecules. The increased energies of these molecules would also increase the probability of their escape from dried preparations. It is postulated, therefore, that the

increased stabilities of dried preparations of virus sealed under helium or hydrogen are a function of the abilities of these gases to lower the numbers of molecules of oxygen, carbon dioxide, and water in the interstices of dried preparations.

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