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

I. Development and Testing of Apparatus*

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(Received for publication, March 8, 1954)

Since the first vacuum dehydration of protein by Shackell in 1909 (1), the technique of drying from the frozen state at reduced pressures has become an important laboratory procedure. This technique (lyophilization) is used today for the preservation of morphological structures, biochemical characteristics, and physiological activities of many biological materials (2–5). Bacteriologists and virologists were among the first to use freeze-drying as a method for preserving infective agents. The various methods of vacuum sublimation employed resulted in dried bacteria and viruses which were, in most instances, viable on rehydration, but little effort was made to obtain maximum survival. Only recently have attempts been made to determine the effects of such factors as speed of freezing and time, rate, and degree of drying on the survival of bacteria (6–9).

Much of the work employing the freeze-drying technique has been carried out with instruments having limited versatility and designed with limited objectives (9–14). Many "freeze-driers" are unsatisfactory for the desiccation of materials maintained below −30°C. during the sublimation process. A vacuum sublimation apparatus suitable for accurate study of the effects of low temperature desiccation on the survival of viruses and cells should be so designed that it will permit (*a*) removal of water molecules at temperatures ranging down to −80°C., (*b*) continuous operation with a minimum of attention from the investigator, (*c*) sealing off samples at operating pressures (10^{-5} mm. Hg), (*d*) the lyophilization of different samples of the same material simultaneously at different temperatures, (*e*) isolation of a portion of the apparatus without disturbing the remainder of the system, (*f*) determination of the end-point of sublimation without removing samples from the vacuum system.

* This study was supported (in part) by grants from the United States Atomic Energy Commission and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

As an initial step in testing the effects of varying the different factors involved in freeze-drying on virus and cell suspensions, we undertook the construction of a molecular still which would meet the above criteria.

Construction of Apparatus

The theoretical discussion of Carman (15) on the subject of the sublimation of ice was used as a basis for the construction of the instrument. The important points are: (*a*) for effective sublimation, the pressure within the system must be less than the vapor pressure of the material at the evaporating surface, (*b*) the vapor pressure of the material at the condenser surface must be lower than the pressure of the system, and (*c*) the temperature of the condenser surface must be such that the accommodation coefficient approaches unity.

Tschudin (16) has shown that the probability of a molecule of water sticking to a condenser (accommodation coefficient) maintained at −60°C. or at lower temperatures is approximately unity. Carman has also pointed out that the mean free path is not a critical factor in the transfer of water molecules from the evaporating surface to the condenser surface. Other workers (17) have shown that the geometry of the system offers little resistance to the transfer of molecules from the evaporator to the condenser surface. However, it must be recognized that the pressure within the system must be of the order of 10^{-4} mm. Hg if the material at the evaporating surface is at −80°C. This pressure (10^{-4} mm. Hg) gives a mean free path of approximately 61.4 cm. Kramers and Stemerding (18) were able to demonstrate experimentally the correctness of Carman's theoretical development. The work of Malmstrom (19) also demonstrates indirectly the validity of Carman's conclusions. The treatise by Dushman (17) on the theories and practices of high vacuum techniques was used as a general reference.

In order to sublimate ice from virus suspensions at −80°C., at which temperature its vapor pressure is 4×10^{-4} mm. Hg, it was necessary to construct a system capable of reaching a pressure of 10^{-5} mm. Hg. To accomplish this, an all glass system using high vacuum stop-cocks and fittings was constructed (Figs. 1 and 2). Apiezon grease was used on all ground glass surfaces.

In order to obtain a pressure in the system lower than the vapor pressure of ice at −80°C., a two stage oil diffusion pump (*ODP*) backed by a suitable mechanical rotary pump, was used. A Pirani gauge (*PG*) was inserted between the fore-pump and the oil diffusion pump. The latter was not turned on until this gauge indicated a pressure in the system well below its breakdown pressure (250 μ of Hg). Pressures of 10^{-5} mm. Hg were reached regularly in the secondary manifolds (*SM*) and 10^{-6} in the primary manifold (*PM*). Narcoil 20, instead of the customary octoil S, was used in the diffusion pump, since this oil does not become tarry and gummy if accidentally exposed to atmospheric conditions at operating temperatures. A mercury diffusion pump was used in an earlier model, but it was found that mercury migrated throughout the system even though a supposedly suitable trap had been placed between

the pump and the manifolds. In order to avoid the deleterious effects of mercury on enzyme systems of biological materials, the change in pumps was made.

The primary manifold (*PM*) was connected to the diffusion pump through a trap (*PMT*). The lower portion of this trap was immersed in a Dewar flask containing dry ice and acetone (−80°C.) as a precaution against back diffusion of oil into the system. During a test run of 10 days the trap showed no sign of contamination with oil. A stop-cock (*A*) was placed in the line between the trap and the pump, so that the latter could be isolated from the rest of the system in the event of a line break.

The secondary manifold was connected to the primary one through a trap (*SMT*). The outer tube of the trap was attached to the secondary manifold through a large stop-cock (*B*). A small stop-cock (*C*) was inserted between the trap and the large stop-cock. This arrangement made it possible to isolate the secondary from the primary manifold and to admit air into this portion without affecting the remainder of the system. The construction of the outer tube of the trap embodied a large, standard taper joint (*D*) which made possible the removal of the lower portion of the trap and the withdrawal of its contents.

The trap was cooled by liquid air contained in a narrow neck Dewar flask of 4.5 liters capacity (*MDF*). This amount of liquid air will last 60 to 72 hours under operating conditions, although in practice the amount lost by evaporation is replaced every 24 hours. The temperature of this trap was −190°C. and the vapor pressure of water at the condenser surface was 10^{-19} mm. Hg.

The female portions of the standard taper joints (*E*) were attached to the secondary manifold. These were arranged in paired sets so that two lyophilization tubes (*LT*) could be immersed simultaneously in a single Dewar flask (*LDF*) and thereby kept at a constant temperature by means of various sludges. The lyophilization tubes were formed by attaching the male portion of the standard taper joint to one end of a section of combustion tubing and a portion of a pyrex test tube to the other end. The heavy wall of the combustion tubing made it possible to seal the lyophilization tubes at operating pressure (10^{-5} mm. Hg). The instrument required only 15 to 30 minutes care per day for checking pressures, replenishing traps with liquid air, adding dry ice to Dewar flasks, etc.

Upon the completion of dehydration the tubes were flame-sealed. During the heating of the central portion of the lyophilization tube, the lower end was immersed in ice water in order to prevent destruction of virus by conducted heat. The sealed vials containing the dried material could then be stored at −40°C.

In order to accelerate the dehydration of materials at temperatures below −50°C., it was found desirable to shorten the distance from the evaporator surface to the condenser surface. The auxiliary system constructed for this

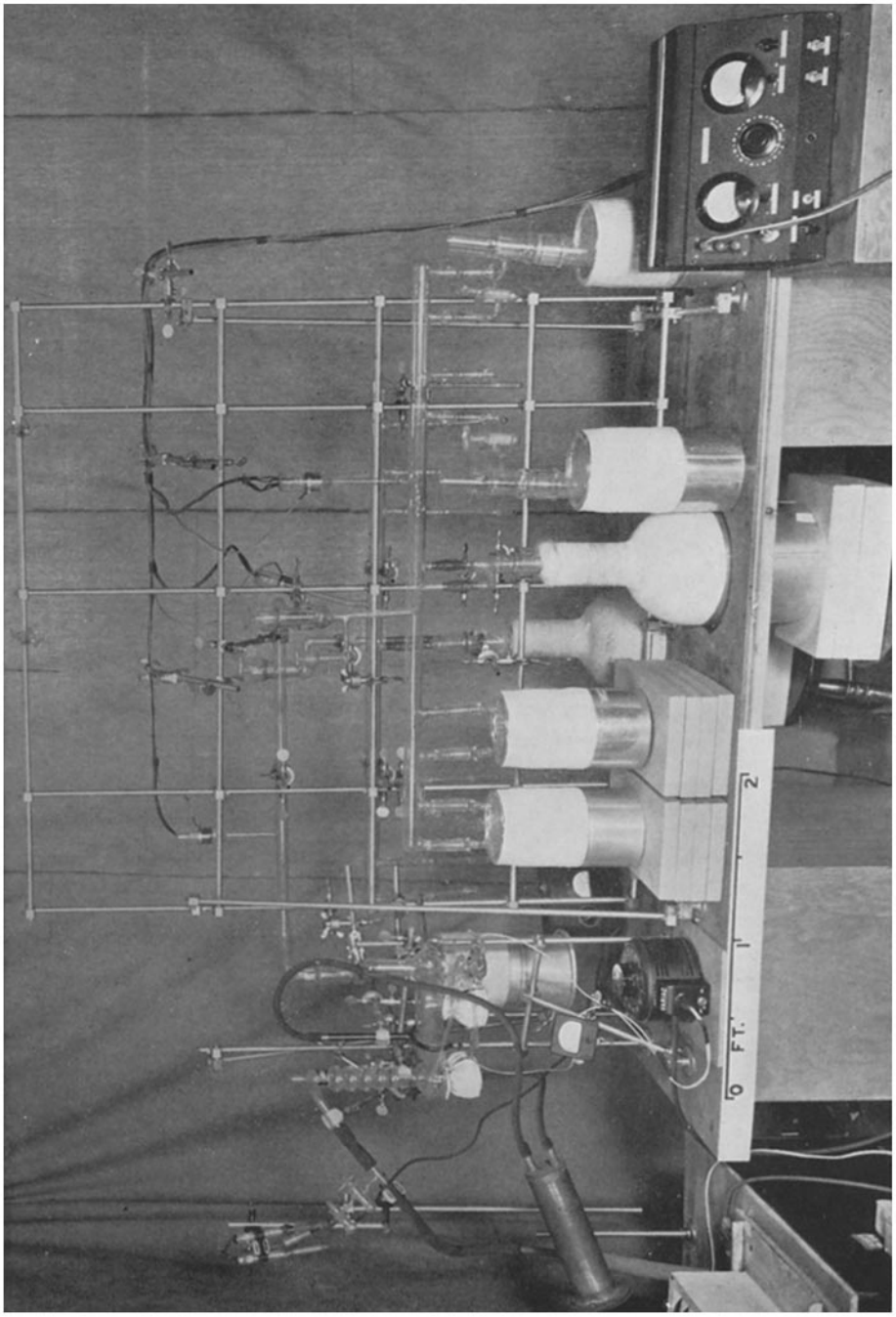

FIG. 1. Apparatus for drying by vacuum sublimation.

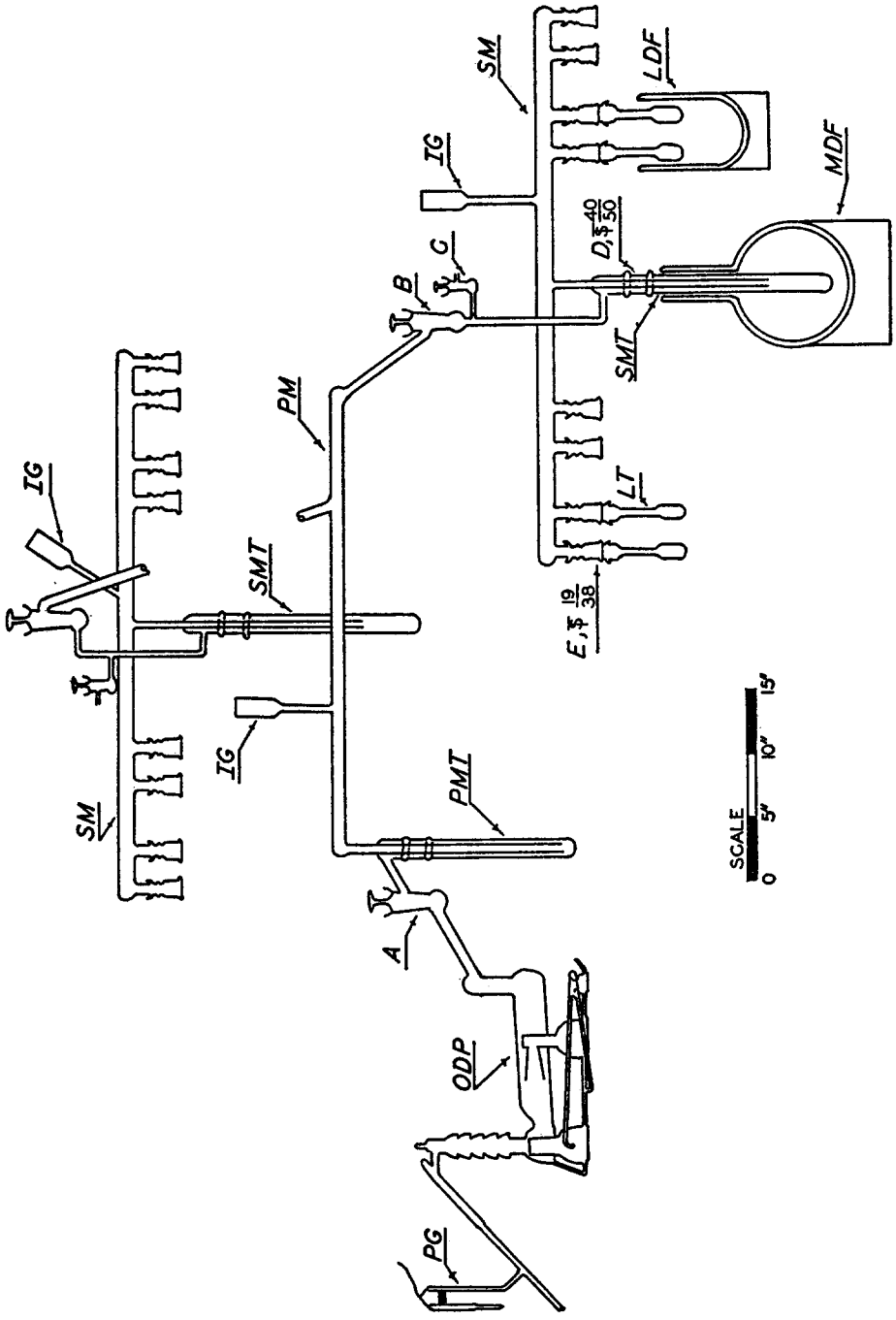

FIG. 2. Diagram of apparatus. The symbols used are given in the text.

purpose is shown in Fig. 3. The material to be dried was placed in the bottom of the outer tube (*OT*) and frozen. This tube was joined to the inner tube (*IT*) by a large ground glass joint and the whole assembly attached to the secondary manifold. A large high vacuum stop-cock (*SC*) was placed between the auxiliary system and the secondary manifold so that a good vacuum could be maintained without sealing-off. The lower portion of the outer tube was immersed in a Dewar flask filled with a sludge at the desired temperature. On reaching a pressure of 10^{-5} mm. Hg in the secondary manifold, the inner tube of the attached system was filled with liquid air. The water molecules leaving the virus suspension condensed on the outer surface of the inner tube

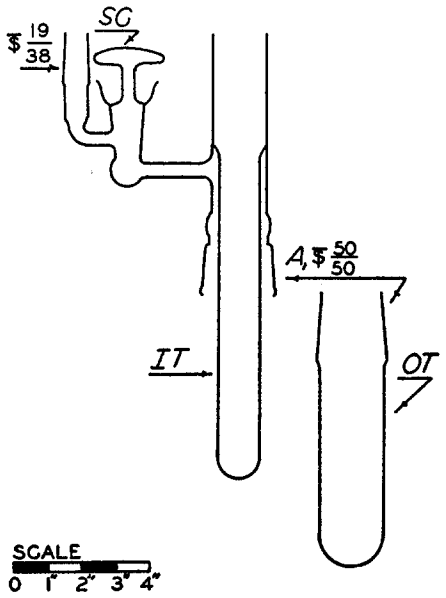

FIG. 3. Auxiliary system used to shorten drying time of virus suspensions.

and remained there until the liquid air had evaporated. The inner tube slowly approached room temperature and the entrapped molecules were freed, and migrated to the secondary manifold trap. The cooling-warming cycle of the subsidiary trap required 3 hours. The periods required to dehydrate 0.3 ml. of the virus suspension at −80°C. with and without this trap were 2 and 8 days respectively.

Ionization gauges (*IG*) were attached to the primary and secondary manifolds. These gauges were used to determine the pressures within various parts of the lyophilizer. The leads from the gauges were brought to terminals in a single panel. It was then possible to energize each gauge and read the pressures in the several parts of the system by switching the control circuit leads from terminal to terminal.

Testing of Apparatus

In a series of preliminary experiments the possibility of using the ionization gauges to determine the end-point of sublimation was explored.

0.2 ml. of distilled water was added to each of several previously weighed lyophilization tubes. The ends of the tubes were stoppered and the water shelled on the sides of the lower ends of the tubes at −80°C. These were then attached to the freeze-dry apparatus, and

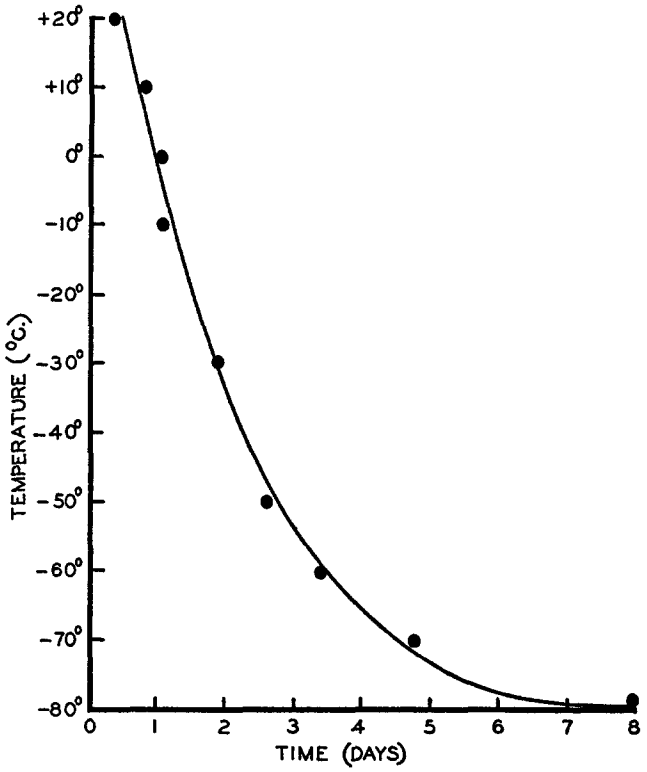

FIG. 4. The effect of temperature on the drying time of 0.1 ml. of influenza A virus suspension.

sublimation at −30°C. started. The tubes were removed, stoppered, and weighed at intervals. The pressure in the system was noted immediately prior to the removal of the tubes. The primary manifold ionization gauge, used as a reference gauge, registered 10^{-6} mm. Hg approximately 24 hours after the pumps were started.

At this time the pressures in the secondary manifolds were too high to be read by means of the ionization gauges ($>10^{-3}$ mm. Hg) and the tubes were heavier than when weighed before the addition of water. 24 hours later secondary manifold pressures were approximately 10^{-3} mm. Hg and the tubes, although weighing less than the previous reading, were still heavier than starting weight. Pressures in the secondary manifolds were then read periodically and the weights of the tubes determined. Both fell steadily. Approximately 60 hours from the start of the observation period, the pressures in the secondary manifolds reached

basal conditions (10^{-5} mm. Hg) and the weights of the lyophilization tubes were not measurably different from their initial weights. Thus the ionization gauges could also be used to follow the course of dehydration.

The time periods required for 0.1 ml. of influenza A virus suspensions (allantoic fluid) to reach an end-point of dehydration at various temperatures are shown in Fig. 4. When larger volumes (0.3 to 0.5 ml.) were used, the shape of the curves remained the same, but a 2- to 4-fold increase in drying time was necessary.

SUMMARY

A vacuum sublimation apparatus is described which will permit, (*a*) the removal of water from virus suspensions at temperatures ranging down to −80°C., (*b*) continuous operation with a minimum of attention from the investigator, (*c*) sealing off of samples at operating pressures (10^{-5} mm. Hg), (*d*) simultaneous lyophilization of aliquot samples at different temperatures, (*e*) isolation of a portion of the apparatus without disturbing the remainder of the system, and (*f*) determination of the end-point of sublimation without disturbing the samples.

The time required for drying 0.1 ml. of influenza virus suspension was shown to increase markedly with decrease of temperature, 8 days being required for dehydration at −80°C. in contrast to 2 days at −30°C. and 1 day at 0°C.

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