Sublimation Freeze-Drying without Vacuum

Abstract. Analysis of the freeze-drying procedure shows that the passage of water vapor from the drying boundary through the dried shell is facilitated primarily by the vapor pressure gradient rather than by the absolute pressure of the system. This is experimentally confirmed, and a device for freeze-drying at atmospheric pressure is described.

The conventional freeze-drying apparatus embodies a specimen chamber with some means of maintaining constant specimen temperature, a pumping system to produce a high vacuum in the specimen chamber, and either a cold trap or a desiccant to remove water vapor evolved from the specimen. The maintenance of a predetermined specimen temperature is necessary to insure that the specimen receives sufficient heat to support sublimation but that its temperature does not rise sufficiently to permit ice-crystal growth or even melting. The purpose of the cold trap or desiccant is to remove from the system water vapor released by the specimen and to prevent the condensation of water in the vacuum pump. However, the function of the high vacuum is not as self-evident as it might at first appear to be, and studies of the mechanism of freeze-drying have led us to means by which the vacuum system may be altogether eliminated.

When a biological specimen freezes, most of the water is removed from solution and isolated in ice crystals, the size of which depends on the rate of freezing. The purpose of subsequent freeze-drying is to remove the water from these crystals without permitting further changes in the specimen. The rate at which water molecules leave the crystal is dependent solely on their temperature. The proportion which return to the crystal depends on the concentration of the surrounding vapor. The net removal of water from the crystal thus depends on the effectiveness with which vapor is prevented from returning to the crystal.

There are two major obstacles to the removal of water vapor: the resistance of the already dried shell of the specimen and any impediment to the transfer of vapor molecules from the specimen surface to the vapor trap. Both theoretically (*1*), and as shown by the experiments described below, the resistance to diffusion created by the dried specimen shell is very great—so great, in fact, that the presence or absence of gas molecules in the spaces vacated by ice crystals is of secondary importance. The factors determining the rate of diffusion of water vapor through this shell are then, (i) rate of vapor production (specimen temperature), (ii) resistance of the dried layer, and (iii) water-vapor pressure at the specimen surface. Since specimen temperature and size are fixed by other considerations, the primary goal in freeze-drying is the efficient removal of water vapor from the specimen surface.

In a system employing a cold trap or desiccant, the purpose of the vacuum is to facilitate the passage of water vapor from the specimen surface to the trap by reducing the number of gas collisions made en route. Ideally, the trap should surround the specimen, and the mean free path should be long enough so that most vapor molecules can travel directly to the trap without collision. Since the rate of passage of water vapor through the dried specimen shell is controlled primarily by water-vapor pressure at the surface of the specimen rather than by total gas pressure, prompt removal of water vapor once it reaches the specimen surface is mandatory for efficient drying. The vacuum system is useful only to facilitate the transfer of vapor to a vapor trap.

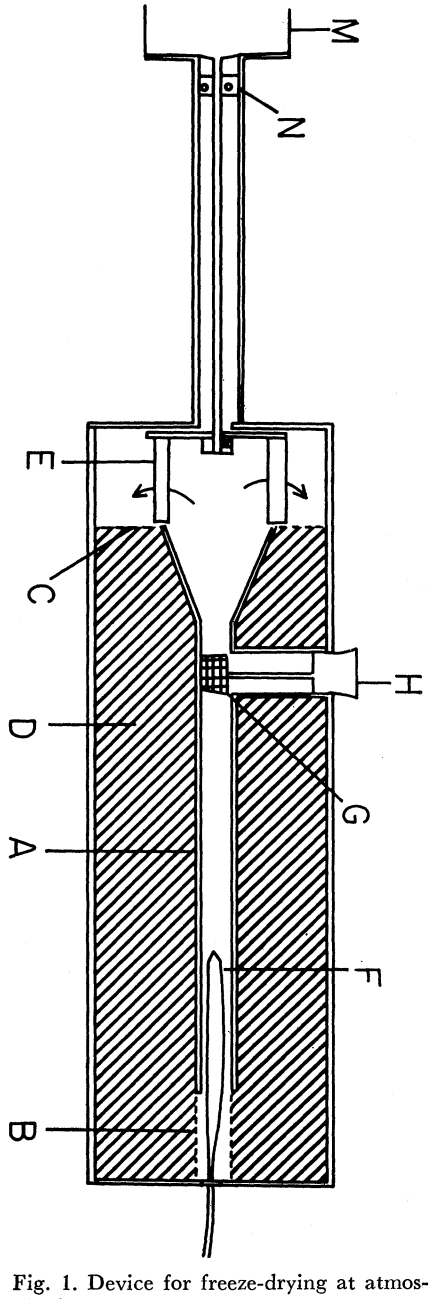

Fig. 1. Device for freeze-drying at atmospheric pressure.

From the foregoing discussion it is clear that any means of removing water vapor from the specimen surface should be nearly as satisfactory as the high-vacuum approach. To this end we have employed dry air blown past the specimen to sweep away water-vapor molecules as they reach the surface of the specimen. A similar approach has been reported by Treffenberg (*2*), who employed a slow transfer through the specimen chamber but in conjunction with reduced pressure. At atmospheric pressure efficiency of the system is improved by increasing the rate of air transfer past the specimen to reduce the thickness of the boundary layer, which will be rich in water vapor.

The apparatus employed is a recirculating system in which air is blown sequentially through a desiccant and across the specimen. The details of construction are shown in Fig. 1. The apparatus is basically a cylinder with a tube running down its center. The specimen is located in a basket *G*, inserted through a port into the central tube. After insertion the system is sealed by stopper *H*. A conventional squirrel-cage blower *E* draws air through the central tube past the specimen and back through the desiccant *D*, which fills the annular space surrounding the central tube. The motor *M* drives the blower through a sealed bearing *N* so that the entire system, once assembled, is completely airtight. The desiccant, retained by screens at *C* and *B*, is a material of very low vapor pressure and high water capacity, properties essential for this purpose (*3*). The apparatus can either be placed in a cold chamber or wrapped with tubing and cooled by a conventional refrigeration system. A thermocouple at *F* permits accurate control of air temperature.

Our standard specimen for the evaluation of freeze-drying techniques is a cube of mouse kidney measuring 2 mm on a side, fresh-frozen by immersion in liquid propane at −195°C and dried at −30°C. A higher temperature of drying permits excessive ice-crystal growth, which impairs the histological integrity of the specimen. From our experience and that of others (*4*), such a specimen requires a minimum of 6 hours' drying time in an efficiently designed vacuum freeze-drying system; a shorter period of time leaves the specimen undried at the center. Standard specimens desiccated in the dry-air device described

above can be dried in 8 hours. Following this drying period the specimen is removed to a vacuum chamber for embedding in paraffin. The quality of the results, in terms of histological detail, appears equivalent to that of results obtained by the conventional vacuum freeze-drying approach. The efficiency of this method compares favorably with the best reported vacuum freeze-drying and is substantially superior to that of devices which do not have the cold trap in line of sight from the specimen.

The mechanical simplicity achieved through elimination of vacuum pumps and use of a vacuum-tight system is considerable. An additional advantage of this approach, however, appears to be in situations where a number of specimens are to be dried simultaneously, where the many specimens and their supports constitute physical obstacles which diminish the effectiveness of the vacuum system by preventing straight-line passage of vapor to the trap. Above all, these experiments (*5*) confirm the supposition that passage of vapor through the dried specimen shell is primarily a matter of vapor pressure gradient rather than of total pressure in the system.

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References and Notes

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2. L. Treffenberg, *Arkiv Zool.* 4, 295 (1953).
3. The material used was Molecular Sieve, a product of Linde Co., a division of Union Carbide, New York, N.Y.
4. D. Glick and D. Bloom, *Exptl. Cell. Research* 10, 687 (1956).
5. I wish to acknowledge the skilled assistance of Raymond Long in the design and construction of the device described in this report.

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Photodynamic Inactivation of Monkey Kidney Cell Monolayers

Abstract. Monkey kidney cell monolayers exposed to white light in the presence of neutral red (1:40,000) undergo degeneration within 24 hours after exposure.

This communication concerns the effect of white light on monkey kidney cell monolayer cultures in the presence of the vital stain neutral red. In the course of attempts to photoreactivate ultraviolet-light-inactivated monkey kidney cell monolayers grown in 100-mm petri dishes, it was observed that unirradiated monolayers, when exposed to white light in the presence of the vital stain neutral red (1:40,000), degenerate within 24 hours after exposure. Similar plates exposed to the same light for the same time period, but in the absence of the neutral red, showed no ill effects from the exposure. Neutral red applied to the cells after exposure to the white light did not produce cell degeneration and was picked up by the cytoplasm as in unexposed cells. If the light was first passed through a solution of neutral red (1:40,000), the particular wavelengths of light responsible for the damage were filtered out, and damage to the cells was prevented. However, when methylene blue (1:10,000) or trypan blue (1:10,000) were used as filters, damage occurred as it did with unfiltered white light. The results are summarized in Table 1.

Table 1. Tissue degeneration with various dyes applied to the culture.

Time of exposure (min)	Tissue degeneration*			
	No dye	Neutral red (1:40,000)	Trypan blue (1:10,000)	Methylene blue (1:10,000)
0	0	0	0	0
2.5	0	0	†	†
5.0	0	0	†	†
10	0	1	0	0
20	0	3	†	†
30	0	4	0	0
60	0	4	0	0
90	0	4	†	†
Light filtered through trypan blue				
60		4		
Light filtered through methylene blue				
60		4		
Light filtered through neutral red				
60		0		

* Degeneration: 100 percent, 4; 50 to 75 percent, 3; 0 to 25 percent, 1; none, 0.
† Not determined.

The source of the white light was a bank of three 20-watt fluorescent bulbs at a distance of 15 cm. The light was filtered through a 3-cm solution of 1-percent copper chloride, and the temperature of the air above the solution did not rise above 38° to 39°C.

It is suggested that perhaps one cause for the occasional degeneration of monkey kidney monolayers or for the loss in plaque count (*1*) seen after the addition of the nutrient-agar–neutral-red mixture used in the Dulbecco Plaque Technique is inadvertent exposure to white light. It is evident from these findings that in the presence of neutral red, the exposure of the tissue to white light should be kept to a minimm.

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References and Notes

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Effects of Differential Infantile Handling upon Weight Gain and Mortality in the Rat and Mouse

Abstract. Animals were handled during ages 1 to 10, 11 to 20, or 1 to 20 days, or were nonhandled controls. Animals handled for 20 days weighed the most in adulthood, while the controls weighed the least. Animals handled on days 1 to 10 survived food and water deprivation the longest of any group. Mice handled for 20 days died earlier than controls, while the reverse was true for the rat.

The general procedure followed in studying the effects of infantile experience upon adult behavior has been to stimulate the organism from birth until weaning and then test for effects of the stimulation later in life. Since the rat and mouse undergo tremendous developmental changes during the pre-weaning period, it is likely that the same stimulation affects the organisms differently at different developmental stages. This is suggested by Scott's critical period hypothesis (*1*) and by the work of Levine and Lewis (*2*). Therefore, if animals are stimulated only during certain parts of the pre-weaning period, the stimulation may interact with maturational processes which are at different stages of development to differentially modify adult behavior.

Complete litters of rats descended from the Harvard Wistar strain, and C57BL/10Sc mice were randomly assigned to one of the following infantile experience groups: handled on days 1 to 10, handled on days 11 to 20, handled on days 1 to 20, and nonhandled controls (*3*). At least two litters were used per experimental treatment. Handling consisted of removing the pups from the home cage, placing them in a container (a 1-gal can filled with sawdust for the rat, and a wooden mouse box for the mouse) where they remained for 3 minutes, and then returning them to the home cage. This procedure was followed once daily on the appropriate days. All animals were weaned at 21 days and reared thereafter with like-sexed members of their own litter in small groups. Food and water were always available. At 69 days the rats were weighed and placed on total food and water deprivation in individual cages. The same was done with the mice at 54 days. All animals had received 10 days of testing of avoidance learning just prior to this. Hours until death occurred were recorded.

The group means for weight and mortality for both species are presented in Table 1. The data were analyzed in a 2×2 factorial design: presence or absence of handling on days 1 to 10 was one factor, while presence or absence of handling on days 11 to 20 was the