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FREEZE DRYING

(PART II)*

By Y. Marzuk

Pharmacist, Public Health Laboratory
(Virology), Tel-Aviv - Jaffo

Harokeach Haiyri, (Hebrew Pharmacist), 9 (10): 1963, pages
605-613

E. The Vacuum

Air Pressure: like any other substance, air has weight which is expressed by its pressure. Men, fauna and flora, inanimate matter and every living cell are in a state of equilibrium in relation to air pressure. Any change in the external pressure causes disturbances in a living system that was adjusted to air pressure.

Air pressure at sea level is 1.03 kg. per cubic cm.

Vacuum: any reduction of air pressure causes a partial vacuum, thus there is a simple method of creating such a vacuum.

* See Part I of article in Harokeach Haiyri, vol. 9, 1963, page 405.

Characteristics of a vacuum system:

1. Hermetic closure
2. Appropriate structure (that can withstand vacuum conditions).

Characteristics of pumps:

1. Piston pump - because of the pistons the vacuum achieved is not high.
2. Rotative pump - the vacuum attained is high, but output is limited.
3. Diffusion pump - tremendous output, but limited vacuum.

See illustrations B (1) (2) (3).

Pumps of various types are therefore combined to attain a high vacuum.

Vacuum measure = Torr = 1 mm. mercury = $1/760$ air pressure. 1 gram of water = 1 mm. in normal temperature and pressure. Under pressure of one Torr per one gram of water per liter volume, that is volume X 1000. [sic]

Instruments for vacuum measurement: there are various instruments for vacuum measurement. They operate in various ways, but they have in common the problem of establishing an appropriate measurement of the vacuum, to the required degree.

Precise calibration of these instruments is done by comparing them with one standard. One of the most accepted standards is the vacuum measurement according to McLeod's gauge (see illustration C); this gauge is based on Boyle's Gas Law and operates as follows:

A certain gas is entered in it and then vacuum is applied to it; the mercury rises in the glass tube in the gauge and the pressure can be determined by the scale.

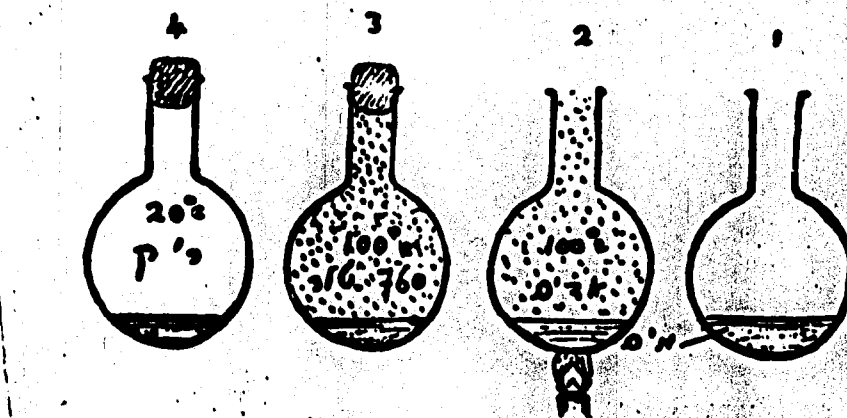


Illustration A: A Simple Method Of Producing Vacuum

1. Vessel with water
2. Boiling
3. Sealing of the vessel
4. Vacuum attained through cooling

Key: 1) Water; 2) Steam; 3) Torr; 4) Vacuum

The calculation is done by these formulas: volume X pressure = volume¹ X pressure¹

$$PV = P_1 V_1$$

$$P = \frac{P_1 V_1}{V}$$

$$V = \pi r^2 \cdot H$$

$P_1 = \Delta H$, if we ignore the system's pressure which is zero.

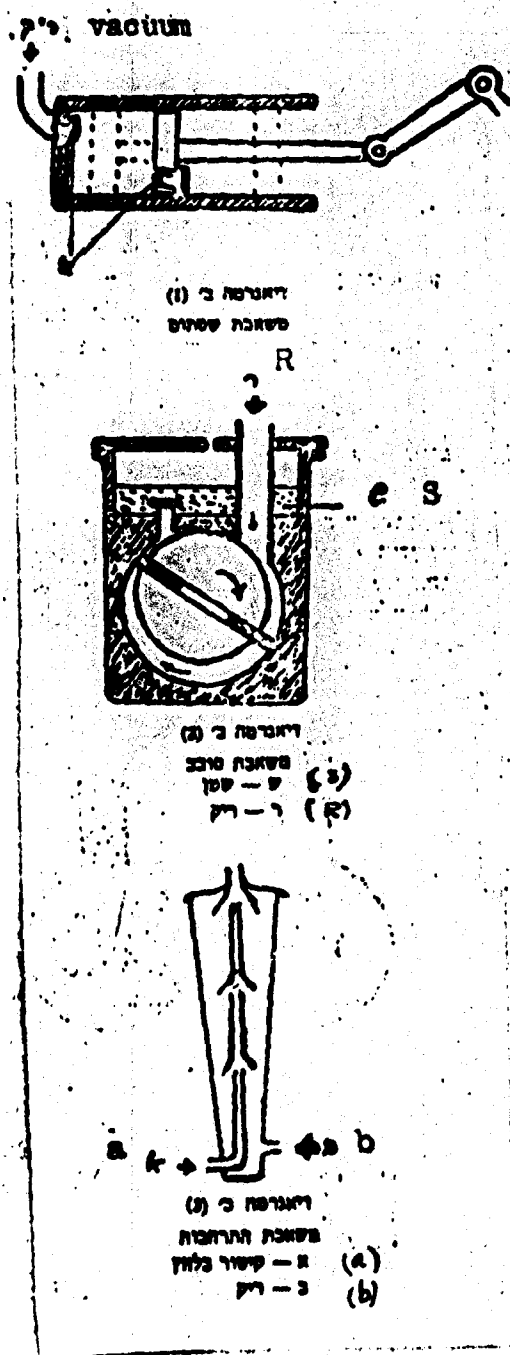


Illustration B.

1. Piston pump
2. Rotative pump where S-oil, R-vacuum
3. Diffusion pump
 - a - steam under pressure
 - b - vacuum

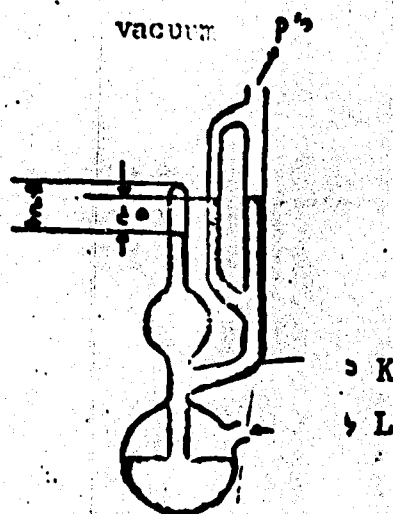


Illustration C: Vacuum Gauge after McLeod

K - mercury
L - atmospheric pressure

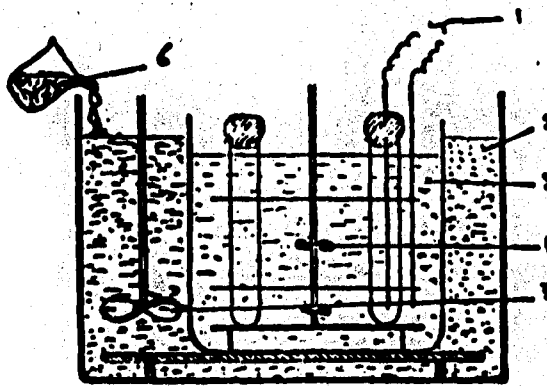


Illustration D: Instrument Providing the Necessary Cooling Rate

1. Thermocouple
2. Alcohol bath - dry ice
3. Alcohol bath
4. Mixer, 800 rotations per minute
5. Mixer 1000 rotations per minute
6. Mixture of dry ice with alcohol

$$P = \frac{\pi r^2}{V} \cdot H \cdot \Delta H$$

which is the McLeod formula, where

r = half the diameter of the glass tube.

V = volume in mm^3 of the glass ball and the tubes.

H = the height in mm in the small closed tube above the mercury.

ΔH = the difference in the mercury height in the small glass tube.

The first two parameters r and V are constant in each instrument and may be obtained from the instrument manufacturer.

The accuracy of this instrument is determined by the accuracy of the measurements of H and ΔH .

The greater the vacuum, the harder the measurements (a fraction of a mm), and accuracy diminishes.

F. Cooling by the Drying System

The theory is that cooling can be achieved through heat-requiring operations, such as endothermic phenomena:

1. Evaporation, which is used most often
2. Release of gas from high pressure.
3. Melting of ice or evaporation of dry ice.
4. Dissolving certain salts such as hydrated salts.
5. Combination of both melting and dissolving.
6. Electrical, magnetic phenomena.

Cooling Through Freeze Drying: #2 mentioned above is suitable - release of gas from high pressure. There are two stages in such a system:

- a. pressure: condensation (creation of heat).
- b. release of pressure: evaporation (heat absorption), in other words, cooling.

Dry Ice

Carbon dioxide in solid state is called dry ice. Under normal atmospheric conditions this ice passes directly from the solid into the gaseous state.

The sublimation temperature at normal pressure (1 kg/cubic cc) is -78.9°C ; its advantages over normal ice are:

1. lower temperature;
2. no liquid residue;
3. it is not chemically active.

In order to assist the movement of cold from the dry ice to the material to be cooled, a liquid is used with a freezing point lower than -80°C , such as alcohol or acetone.

Liquid Air

Liquid air is produced by a process of compression and cooling. In the first stage a pressure of 40 kg is applied to air and later the compressed air is transferred for cooling to a compressor of 200 kg.

Lindeh was the first to produce liquid air on an industrial scale in 1896.

The compressed air at a pressure of 40 kg is partly used for cooling (up to -100°C), and the rest is passed to the second compressor where a part of it is used for cooling (-160°C) ... (-190°C) and so on till the end when -210°C is reached.

Accepted Mixtures for Cooling

1. Table salt with plain ice, -20°C
2. Potassium chloride with plain ice, -40°C
3. Alcohol with dry ice -70°C
4. Ethylene glycol with dry ice -70°C
5. Liquid air -196°C

G. Biological Stability

Biological matter is usually unstable. If the diphtheria toxin are taken as an example we see that its toxicity is diminished with time, especially when the temperature rises.

The change in toxicity is a result of a change in the chemical composition of the toxin. The rate of change of the chemical structure of the toxin is slower at lower temperatures, but slowing down the rate of such changes does not continue in sub-zero temperatures. When the freezing point is passed, the molecular order is changed, which may damage the toxin, but when the eutectic temperature is quickly passed stability of the toxin may be attained.

Therefore, one of the methods of preserving biological materials is freezing, since low temperatures slow down the spoiling processes of these materials, when a quick change beyond the eutectic temperature of that material is achieved. If, after freezing, drying is applied, a peak in the stability of the substance is attained.

An example: the activity of the Cobaye complement is reduced to half within three hours at room temperature, within 24 hours at a temperature of 1°C ; within three months at -10°C ; within a year at -25°C ; and can be preserved indefinitely at -80°C .

As mentioned above, the shift beyond the eutectic temperature must be fast in order to prevent concentration of salts which causes denaturing of proteins.

The freezing of cells requires delicate handling and constant observation.

Two factors determine the condition of the freezing:

1. Cell viability.
2. The structure of the cell's membrane.

For example - the red blood corpuscles have a very delicate membrane, and sperm cells have strong membrane and deficient viability (frozen sperm cells may remain intact, but their mortality is high); microbes are in between these two extremes.

Parks discovered that bull's sperm loses its viability because of the thermal shock. In fast cooling from 15°C to zero there is high mortality, but with slow cooling at the rate of 5°C every 20 minutes in the presence of egg yolk the sperm cells of the bull will overcome the thermal shock.

Instead of yolk, glycerol or ethylene glycol may be used.

The action of the glycerol is two-fold:

- a. Protection against the thermal shock,
- b. Prevention of crystallization (which is important in the case of cells).

When the red blood corpuscles are frozen in a medium containing 30% glycerol, they remain intact, but it is difficult to remove the glycerol; indeed, it is possible to do this in the laboratory by dialysis, but this method is not practical for blood transfusions.

It was proven that protein may be preserved indefinitely and without change by methods of drying and freezing. The drier it is, the more stable it is.

Practically speaking, a 1% moisture content is an accepted limit for drying, but if the substance is further dried, the protein will be well preserved.

In the case of microbes the situation is different, and a certain percentage of moisture is required to preserve their life.

H. Preservation of Tissue Cultures in Sub-Zero Temperatures

Work on various types of tissues, in biology, and as nutrient in cultivating viruses, involves various problems.

Passage of cells and their growing for a long period increase the effect of the artificial cultivation conditions. These conditions may effect among other things the genetical integrity [mikhlo] or cause mutations and also cause characteristics that these tissues did not have at first.

For instance, normal cells turn into malignant cells, or vice versa.

Such tissues, being rich in biological basic materials, act as nutrients, and thus are constantly in danger of contamination by external micro-organisms.

Even the transfer of these tissues from vessel to vessel every few days (3-7 days according to the degree of their development), requires much and expensive professional work.

Preservation of tissues at sub-zero temperatures solves these difficulties for us.

Such preservation is determined by the following factors:

1. Medium in which the cells are suspended.
2. Number of cells/ml of suspension.
3. Rate of freezing.
4. Temperature of storing.
5. Length of storing.
6. Rate of thawing.

Cell Preparation for Freezing

The Culture: Cells intended for freezing must be vigorous in structure, healthy cells, and they must be in the logarithmic stage of their development curve.

The cells must be examined and must be free of contamination by microbes, viruses and other factors.

It is desirable that the medium where a culture was grown be changed or at least freshened two hours before harvesting.

Harvesting: the cells are collected mechanically, by scratching the culture-bottles, or by means of an enzyme [Tasas] such as trypsin. When an enzyme is used, the cells must be washed later or a considerable amount of serum added to counterbalance the action of the enzyme.

Suspension Medium: the medium with an additive or preservative such as glycerol or dimethyl sulfoxide in a concentration of 5-15% by volume.

Since one of the glycerol's characteristics is to penetrate the cell's membrane, and there is a difference in the membranes of various cells, it is possible to distin-

guish between the various cells by their toleration of glycerol. The toxic effect of glycerol on cells increases with heat, and therefore it is desirable to add the glycerol at 4°C.

The results with the use of dimethyl sulfoxide are not yet known.

For unknown reasons the rate of survival rises in fibroblastic cells when the concentration of preservative is low: in epithelial cells it rises when the concentration is higher.

Number of cells per ml of suspension: the most adequate concentration of suspension for freezing purposes ranges between 1-5 million cells per ml. Care must be taken that the cells won't sink in the medium during freezing.

Storage - it turned out that it is better to seal the glass test-tubes where the cells are in suspension by melting the glass on top in order to prevent escape of carbon dioxide and a change in pH, as well as to prevent other changes such as oxidation. Other methods of sealing with rubber or corks are not hermetic enough.

The cooling Rate: a suitable cooling rate is 1°C per minute (Fogel, Smith and Parks). One of the simple and efficient tools for assuring the required rate of cooling is used by the tissue bank of the U.S. Navy (see illustration D). There are also more complicated instruments for laboratory and industrial use.

Storing: with the help of an adequate freezer or dry ice, temperatures of -60°C to -70°C are achieved; liquid nitrogen -196°C, is also used.

Shriver and his associates succeeded in preserving cells for 6-7 months by freezing with a survival rate of 30%-50%.

In this connection, and considering also the ability of the cells to cling to the panels and create a tissue, and not only the survival ability itself, it was found that out of cells stored for a period of 3-4 years, up to 50% living cells were obtained after thawing, but their ability to develop a tissue or a mat (marvad) on the panels was impaired. This problem requires further study.

Thawing: by immersion in a water bath at 37°C a larger number of living cells was obtained than by other methods.

References:

- 1) Scheres, W.F. and Hoogasian, A.C., Preservation at Sub-Zero Temperatures of Mouse Fibroblasts (Strain L) and Human Epithelial Cells. (Strain HeLa) 1954, Proc. Soc. Exp. Biol. & Med., 87, pp 480-487.
- 2) Swim, H.E., Jaff. R.F. and Parker, R.F., Some practical aspects of Storing Mammalian Cells in the Dry-Ice Chest. 1958, Cancer Research 18, pp 711, 717.
- 3) Stevenson, R.E., Preservation of Cultural Cell Lines at Low Temperatures 3rd Int. Freeze-Drying Course, 1962, Lyon.

THIRD PART

I. Drying of Living Organisms

The main factor affecting the percentage of survival after freeze-drying when the medium is immediately returned to its former state, or after prolonged storing, is the medium in which the organism is suspended.

In 1949 Greaves and associates (Fry and Greaves) proposed a medium containing 75% serum and 25% broth containing 7.5% glucose. In this medium a high number of living organisms were preserved, including delicate organisms such as *Neisseria gonorrhoeae* and *Vibrio cholerae*, and their opinion was that the serum is protective colloid and prevents the dried matter from crumbling, and that the glucose preserves the required moisture.

Such a medium, of course, is not suitable for all purposes; the presence of the serum, for instance, does not make it possible to inject it into humans.

In 1960 Obayashi used sodium glutamate to increase the stability of the tuberculosis vaccine, B.C.G. at high temperatures, and found that glucose counterbalances the effect of the glutamate, and sucrose does not.

Therefore it was proposed that the tuberculosis vaccine which is intended for storing in people [sic] be dried in a medium containing dextran free of glucose, 1% of sodium glutamate and 50% sucrose. The use of dextran came after it was proved that it does not act as an antigen, hence it replaces the serum; the sodium glutamate fills the function of the broth in the mentioned medium, and the sugar preserves the percentage of moisture required for survival.

The same year (1960) Scott approached the problem from another direction. He dried the cultures in a broth containing papain digest and preserved them in various concentrations of active water* with the help of various drying salts at various temperatures and found that at low concentrations of active water and at low temperatures life survived when glucose was present. Such dried cultures were not well preserved at high temperatures; on the other hand the sucrose was more active at high temperatures.

Scott concluded from his experiments that the stability of the cultures at high temperatures is caused by the presence of carbonyl group $C = O$ (Ketonic group); the amino acids in Greaves' broth counterbalanced the carbonyl group; glucose, instead of sugar, added a carbonyl.

The criteria of a medium adequate for drying are that it will make an immediate and high survival possible, and that it could be preserved for long periods at room temperature. Accordingly, Greaves introduced an experimental method for examining the dry material by heating it to $100^{\circ}C$, and measuring the time during which the viable count decreased to one half. Under these conditions it was found that a medium containing 5% peptone preserves well for a long period of time, which strengthened Scott's hypothesis.

Sodium glutamate is most effective at a concentration of 5%; at a lower concentration the immediate survival is low, which indicates lack of moisture. At high concentrations sodium glutamate acts in two directions:

* See water classification under "Drying of Living Cells"

- a. it counterbalances the free carbonyl group;
- b. it buffers the residual moisture.

The activity of this sodium glutamate is improved when colloid (5% dextran free of glucose) is added.

Greaves summed up his work in 1960 stating that a medium for the drying of microbes must contain:

1. preservative colloid such as 5% dextran free of glucose;
2. a residual moisture of around 1%, sucrose at a concentration of 5-10%, or sodium glutamate 5-10%;
3. material that counterbalances the carbonyl group such as broth or 1% sodium glutamate.

In 1961 Obayashi and his associates proposed the use of polyvinyl pyrrolidone - P.V.P. - instead of dextran.

It is interesting that in 1955, Collier recommended only peptone 5% as a preservative material to dry the Vaccinia virus, and it was proved later that this percentage is even more suitable for other viruses such as the Influenza virus and the Herpes Simplex; on the other hand it is not suitable for preserving microbes since they became too dry.

Is there a connection between the amount of moisture required for the life of the organism and its position in the life scale? Research is already being carried out on this question.

J. Drying of Living Cells

Attempts at reviving cells of mammals were far from successful until blood was first frozen without glycerol. A medium that contained glycerol for that purpose caused many complicated problems.

Success in the dry freezing of red blood corpuscles was attained after many failures. When red corpuscles are dried by the usual methods they appear under the microscope as bright rings having a crystal structure. When an isotonic salt solution is added to such a dried red corpuscle in order to bring it back to the original state, the natural shape changes immediately into a spherical shape, and then the red corpuscle explodes.

This form of crumbling is typical in osmotic mal-adjustment; crumbling is not prevented even when solutions with high osmotic pressure are used. Even a solution of 50% sugar won't change the result.

A better result is obtained in preserving red corpuscles when the restitutive solution is an isotonic-salt solution containing 40% dextran.

A Simple and Basic Method for Drying Living Cells:
A suspension of red corpuscles is put in a spray-drying vessel. The spraying creates tiny droplets containing the red corpuscles; a vacuum created by suitable pumps is applied to the drying-vessel. The vacuum causes evaporation of 10-15% of the suspension's water droplets, which causes cooling, all the way to freezing of the droplets. Later in the drying (evaporation) process the temperature continues to go down till it reaches -30°C .

This temperature level is the result of the loss of calories through evaporation and absorption of calories through radiation from the environment.

When the evaporation rate is slowed by the progress of drying, the temperature rises to -25°C within 2 to 3 minutes from the freezing time of the drops, finally the temperature rises quickly to room temperature.

The Appropriate Rate of Drying: mammalian cells after their drying contain about 8% residual moisture. When red corpuscles are dried by the spraying method described above, there is about 100% recovery. Drying of red corpuscles beyond the mentioned percentage (8% residual moisture) considerably reduces the percentage of whole cells recovered after their wetting. This loss is not effected by the composition of the restitutive solution.

In this connection, Meryman assumes that drying in which an 8% residual moisture is reached is similar to the freezing condition where the relatively free water, such as ice crystals, is separated; this condition does not hurt the organic structure of the cell, but upon the increase of drying, water is separated that is more closely connected with the cell's molecules, and therefore, has a more vital function in the structure of the molecules that are the components of the cells.

Hypothesis Regarding Storing Cells in Freezing:
Meryman and his associates believe that dextran and P.V.P. have a vital function in preserving the cell during drying.

The cell membrane is composed of lypoprotein, and the cell's transport mechanism is located in the pores of the membrane; he believes that the transport mechanism has a high moisture content and that this transport is carried out through watery liquids.

In the advanced stage of drying the water which is more closely connected to the molecules of the transport mechanism is separated, and then the pores in the cell's membrane open. When the cells are moistened with isotonic solution, increased quantities of salt and water penetrate the cell before the transport mechanism is reconstituted.

Dextran or P.V.P. in the moistening solution provide a protective layer on the cell's membrane and provide a piston for the passages (the pores) until the latter are reconstituted.

The connection between the cell's membrane and these materials (dextran and P.V.P) is an electrophysical one. This relation will not exist in the case of further increase in drying, whence the difficulty in recovery from this advanced stage of drying.

On the basis of these hypotheses the cell water may be classified into three types:

- a. free water - in intra-cellular solutions;
- b. active water, the removal of which stops the cell's activity;
- c. vital water, the removal of which stops the cell's life.

References:

1. Greaves, R.I.N.: Recent Advances in Freeze Drying, Journal of Pharmacy and Pharmacology, 1962, 14, pp 621-640.
2. Meryman, H.T.: Drying of Living Mammalian Cells, Annals of N.Y. Academy of Sciences, 1960, 85, pp 729-734.
3. Rey, L: Herman, Paris, Actualities scientifiques et industrielles.