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Dry Preservation

Air drying

Many of the invertebrates, especially those with adequate exoskeletons, may be air dried. In particular the insects are usually treated in this way after careful positioning and pinning (setting). It will be necessary to take precautions against infestation of air drying material by provision of containers in which the samples can dry without any danger of infection or infestation. A simple technique, merely exposure to ambient temperatures with adequate safeguards against dust, dirt and pest infestation.

Warm and hot ovens

An improvement over simple air drying. Providing controllable heat to speed drying times and increase productivity. It is not usual to exceed a temperature of 60C for this type of air drying.

Sand baths, etc.

Metal trays are normally employed for this type of drying. A measured quantity of fine grade sand is poured to a constant level in the tray. A similar amount of sand is heated to 60°C in an oven. The sample to be dried is gently pressed into the surface of the sand in the tray and the heated sand poured over the specimen to completely cover it. The bath is left for 12 hours and the dried specimen removed by gently pouring away the covering sand. A modification of this process utilises a tin can with a narrow screw cap, the bottom of which has been cut out. The can is placed upside down on a support. Sand is poured in to fill half the container and the specimen gently pressed into this as before. The heated quantity of sand is then added and allowed to remain for 12 hours. By unscrewing the cap the sand will drain away with a minimum of movement to the specimen in the can. This is very useful for long delicate specimens, sponges, branching corals, invertebrates with lengthy appendages etc., when and where a sand tray may not be suitable. Silica gel may be used in the same way as sand and there are many grades right down to a fine dust. Colour indicators such as cobalt chloride may be used to monitor the drying. This is done by adding a few grains of the reagent to the sand or silica making sure that the reagent is quite dry before use. The cobalt salt is a deep blue when dry and shades of red when moist. Baking in an oven will regenerate it to the original blue colour.

Riker mounts.

Consists essentially of a glass topped box in which layers of cotton wool and sometimes tissue paper are placed. The specimen is pressed into the layers and the glass lid allows a protection and excellent observation. Used for many invertebrate forms.

Chlorocresol mounts.

A more successful modification of the Riker mount in which a layer of chlorocresol is placed at the bottom of the glass topped box and this acts as a preservative. This has been a very successful technique for sending delicate invertebrates through the post or while on expeditions as the form, shape and colour are preserved indefinitely (Tindale, 1962). Very much used in Australasia and S.E. Asia generally. A very useful pre-preservation technique for invertebrates in general when no adequate preserving media are available.

Freeze drying.

History

The sublimation of ice has been known since the early 18th century and was described by William Hyde Wollaston in a communication to the Royal Society in 1813. In 1890, in Leipzig, Richard Altmann described a method for the freeze drying of tissue for histological examination. This ingenious idea went largely unnoticed apart from brief recognition in papers and books by Mann (1902), Bayliss (1924), Mathews (1925) and Romeis (1928). It was not until 1932 that Gersh in Chicago again described the Altmann technique and suggested modifications for further histological work.

In 1939 Kidd in Cambridge took out a patent for freeze drying food involving different principles to those used in the treatment of entire animals and plants. It was in 1954 in this country that Davies wrote a short paper on the preservation of insects by drying in vacuo at low temperatures (Davies 1954,1956). Previously in France Mercie in 1948 had suggested a method of controlled vacuum drying, and in 1959 Stadelman at the International Botanical Congress in Montreal repeated Mercie's work and gave an account of work on fungi.

Meryman in Washington, USA, in 1959 (Meryman 1960, 1961), gave an account of the freeze drying of small animals and birds and some invertebrates by the standard technique that has now been adopted to general use in this field. In 1960 Haskins in Canada showed that macro fungi could be well preserved by freeze drying and this was followed in 1962 by further work by Davies in which he too, described work on the larger fungi. In 1963 Woodring and Blum (1963a,1963b) at Baton Rouge USA, described methods for the treatment of insects and arachnids.

In 1964 Harris in England and Hower in Washington USA wrote accounts of work based on the original work of Meryman. Harris was the first worker in the UK to develop apparatus specifically intended for the treatment of entire biological specimens. He concentrated on small specimens and also on cell integrity for histological investigation. Meanwhile, Hower in America developed bigger apparatus for larger biological specimens having the advantage of apparatus available through the space programme and no longer required for that purpose. In 1968 Harris described a new apparatus

of a portable nature for whole biological specimens and it was the first apparatus to be produced commercially in Europe in which the techniques of vacuum dehydration and freeze drying were combined. Since that time freeze drying has been found useful in Archaeology, Mineralogical and Palaeontological investigations, and also in Fine Art for the prevention of deterioration. It has also been found to be the most practical technique for the preparation of soft tissues for examination using the stereoscan electron microscope.

General account of the freeze drying technique.

It is one of the most efficient methods yet devised for preserving animal and plant material in the dry state, without shrinkage and with the natural colours intact. The technique involves the initial freezing of the specimen followed by the sublimation of the water vapour from the ice crystals of the specimen so that it will progressively lose weight until a final constant weight is arrived at. When this situation has been reached the specimen is considered dry and will not react to moisture indicators although chemically bound water is present in small quantities. It should be noted that water in the liquid state is not present at any time during the process.

Specimens which have been frozen, as indicated above, will when exposed to a low vacuum and temperature give off water vapour which collects in the form of a cloud and hangs as an aura in the vicinity of the specimen. This may be drawn away by the use of a desiccant or a condenser. In the most efficient systems the condenser is maintained at a substantially lower temperature than the specimen so that a temperature gradient forms. This encourages the water vapour molecules to migrate continuously from the specimen to the condenser and so to dry the specimen. The mechanisms through which freeze drying denatures biological constituents are still largely unknown, and a substantial amount of fundamental research is obviously necessary before the full potential of freeze drying can be realised (Rowe, 1960).

Since freeze drying entails the removal, first of free water and then of 'bound water', investigations of the drying process and of the mechanisms of further protection from later breakdown can all contribute to a better understanding of the technique. 'Bound water' is that which does not freeze. It comprises between 5 and 10% of the total water content of a typical animal. There is no sharp distinction between wholly free and totally bound water. Present knowledge suggests that a temperature of -10 to -20°C and a pressure of 10 to 40 microns Hg (0.04 Torr) will remove most freezable water from a cell interior to form ice outside the cell. A condenser at a lower temperature, -40°C, will effectively remove the ice from the cell, which will collect on the condenser surface.

In freeze drying investigations, the surface has barely been scratched and it is hoped that future studies will bring more emphasis on basic questions of

freezing and drying and that the technique will be increasingly recognised as a subject for major fundamental research and not merely a technological curiosity.

Preliminary treatment of animal tissues for freeze drying

Protozoa - The easiest to prepare are the freshwater groups. Relax the specimen in de-ionised water with a crystal of menthol. May take an hour or two to completely relax. Wash away any remains of menthol before the initial freezing to prevent build up of dissolved solids. Use Steedman's multipurpose fixative for several hours and wash out thoroughly.

Steedman's fixative

Propylene phenoxytol	1ml
Propylene glycol	5mls
Formaldehyde	5mls
Distilled water	89mls

Good preparations are well extended, and suitable for surface illumination microscopy or stereoscan electron microscopy. Marine groups must be relaxed in sea water first then transferred to fresh de-ionised water to remove the dissolved solids in sea water. Normal relaxing reagents can be used as required. Terrestrial groups are frozen in small amounts of de-ionised water. They may need treatment with liquid nitrogen to remove the external debris with which many will be coated. Preserved protozoa may be successfully freeze dried if they are first thoroughly washed to remove all traces of the fixative. Special care must be taken when dealing with formaldehyde preserved specimens. Small traces of this reagent can cause damage to the condenser surfaces of the drier, causing expensive replacement repairs. Freeze dried protozoa do not need to be placed in a desiccator as they are not hygroscopic. They are, however, liable to be affected by dust and should be placed in a clean dust free container.

Porifera - These are good subjects for freeze drying. Care is essential to remove all debris and surface contaminants by thorough washing in several changes of de-ionised water. Marine or freshwater forms give equally good results. Colour retention is reasonable if they are treated as soon as possible after collection and before any deterioration has taken place.

Coelenterata - Provided the specimens are properly narcotised and well extended, coelenterates freeze dry very well with good colour retention. There may, however, be many problems with the initial relaxation. It is, therefore, prudent to experiment with *Hydra* and *Aurelia* first before trying rarer, more exotic forms.

Platyhelminthes, Nematodes and Minor Phyla - All groups freeze dry well. Subjects must be clean and debris free before initial freezing. Nematodes are best relaxed in dilute watery iodine (1:1000). Endoprocts need relaxation

in MS 222 before washing. Other groups are best treated as for nematodes. Rotifers are difficult to prepare but the multipurpose fixative used for the protozoa often gives good results.

Annelids - This is a good group for freeze drying, and show good colour retention. They need careful relaxation (MS 222, menthol, Well's technique etc.); special care is necessary with leeches.

Molluscs - Careful relaxation gives very good results. It is often necessary to wash off slime from terrestrial groups before initial freezing to prevent gross contamination with the dried exudate after freeze drying. Asphyxiation in boiled cooled water (to remove the air) has often proven to be the best method of relaxation. Add a crystal of menthol to the water in a closed container, sealed to exclude the air and place in a refrigerator (not a freezer) at 4C.

Lophophores - All groups freeze dry well. With Brachiopods make sure that the valves are not under stress before initial freezing.

Echinoderms - All groups must be washed carefully as this entirely marine family are exceptionally septic and give very nasty infections on abraded skin. Relax in freshwater, which acts as an anaesthetic. Good colour retention.

Arthropods - All groups under this heading freeze dry very well. Care in preparation and layout before initial freezing pays dividends. The only really efficient way to preserve spiders with good colour retention.

Chordates, Tunicates and Ascidians - All groups dry well. Thorough washing out of the seawater and careful arrangement before initial freezing is necessary for acceptable results.

Fish - A difficult group to deal with due to the presence of fat and oil deposits in the skin and organs. Freeze drying does not alter fat in any way apart from sterilising and rendering it bacteria free. Defatting techniques are sometimes successful. Colour retention is often very good until natural oxidation processes cause a slow loss of colour. The most successful method of fish preparation is based on the taxidermist's method, of removing the viscera from one side, then freeze drying the fish to show the other side only.

Amphibia - A less successful group. Some forms dry well with good colour retention. In many cases, however, discolouration of the skin and tissue shrinkage will occur. Much trial and error and the availability of plenty of specimens is needed to get consistently good results. Injection of the body with water prior to the initial freezing may help to prevent the shrinkage. Amphibia with dry skins, such as toads tend to dry better than those with thin moist skins such as frogs. Common newts collected when in their aquatic phase tend to freeze dry badly compared to those collected later when they have left the water.

Reptiles - These are very good subjects for freeze drying. In many cases excellent colour retention is shown. Reptiles share with birds and mammals

the following requirements for the best results. Posing of the killed animal is necessary before drying. The removal of the viscera will accelerate the drying, as will the drilling of small holes in the body of the frozen specimen. These procedures will merely hurry the process and are not otherwise essential. It is usual to replace the eyes with glass eyes before the initial deep freezing as the eyes become opaque during the process. Alternatively they may be painted afterwards, but the appearance, though adequate if skilfully done, is not so satisfactory.

Birds - One of the best groups to deal with. Shows the great capability of freeze drying. Better than many taxidermy preparations. Allows the specialist preparator to use skill and artistry backed up with knowledge of anatomy without the shedding of blood and viscera as all the organs are retained entire, the whole body being untouched. Only the eyes need attention as stated above.

Mammals - Specimens up to the size of a small rabbit are very well dried. Above that size it is usual to drape the skin over a modelled body. The larger specimens can still be processed, however, provided the specimen chamber is large enough and the operator is prepared for a long processing time. From rabbit size downwards all mammals are treated as for the other higher vertebrates, in the entire state.

Anatomical material - Excellent for preserving dissections and whole organs, for general anatomical and also pathology specimens. Such items can form a useful alternative and supplement to fluid preserved specimens in many cases with excellent colour retention. It is often useful to spray such items with a coat of shiny resin partly to prevent the attention of insect pests and also with the purpose to impart to the surface of the specimen a lifelike sheen.

Preliminary Treatment of Plant tissue.

Algae - All this group freeze dry very successfully, usually with good colour retention. It is necessary to pose the specimens either in the herbarium style or in any other natural posture. Time varies according to the size of the specimen.

Fungi - This group will dry excellently, the permeability and high moisture content of these plants make them particularly good subjects for freeze drying. Small specimens may be freeze dried overnight but the larger forms should be weighed at intervals till constant weight is achieved. It is a simple matter to test fungi for completion of drying. Dried forms are very light and feel warm. The slightest coldness indicates that the process is incomplete. Note: The more brightly coloured forms should be stored in the dark for they will fade if exposed to ultra violet light, either natural or artificial, for any length of time.

Mosses and Ferns - These groups are excellent examples for freeze drying and colour retention is good.

Flowering Plants - These groups can give excellent results, although problems can arise in respect of colour retention. Material must be fresh picked preferably with newly opened blooms and should be processed as soon as possible. For the best results with large brightly coloured flowers pre-freeze by standing in a metal container with liquid nitrogen which is poured into the vessel. This is then placed in the freezing chamber of the freeze dryer which should be at a temperature of -20°C. Allow plenty of time to elapse for the liquid nitrogen to evaporate before turning on the vacuum pump. As with the fungi the more brightly coloured forms are best stored in the dark.

General procedure for freeze drying

Before treatment all specimens should be initially frozen at a temperature of -10°C or lower for a period of 12 hours. When entire animals are being treated, it will probably be necessary before freezing to pose the specimen in a desired position. This can be achieved in a variety of ways, arrangements of wire and balsa wood supports may be constructed to display it. Some animals can be pinned into position, cotton wool and plasticine may be arranged to keep the body in a natural shape till frozen. Occasionally shrinkage of the body may occur due to constriction of the muscles. This may be corrected by injecting water into the affected part to fill out the space until a normal appearance is attained. The water will of course will be sublimed away as vapour during freeze drying. Organs which tend to flatten under their own weight after removal from the body so that they present an unnatural appearance after drying may be dealt with as suggested above.

Freeze drying will prepare specimens of plants and animals which are difficult and in some cases impossible to deal with adequately in any other way. Very small birds and mammals for example, may be dried in the entire state, with all their organs intact, except as mentioned above possibly the eyes.

Examples of freeze dried specimens

Reconstituted toad - As a test to ascertain the condition of the cellular integrity of freeze dried tissue a toad was freeze dried and left for a year. The animal was then bisected sagitally and one half left as a dry control, while the other was rehydrated using a 0.5% aqueous solution of tribasic sodium phosphate. When the organs reached a reasonable shape the tissues were placed into fixative. They were then processed by wax impregnation for routine histology sectioned and stained with Mayer's haematein and eosin and Masson's trichrome stain for microscopic examination. All the cells of the organs examined, though somewhat reduced in size, appeared to be normal, and the staining reactions were intensified by the process.

Valuable specimens have been freeze dried including a ***chinchilla*** and ***emperor penguin chicks.*** These specimens were in excess of 2kg in the initial frozen weight.

An ***elephant heart*** weighing 2700gms was dried in six days to a constant weight. Previously it had been preserved in a formaldehyde solution. Freeze drying enabled the specimen to be easily moved about, handled and freely examined without the hazards of bulky glass or plastic containers filled with dangerous fluid.

Mummified human hand - The hand was removed from a Guanche mummy from the Canary Islands. The mummy was pre-dynastic and 15,000 years old. Mummification had been affected by burying the corpse in hot sand. The hand was removed and then reconstituted using a solution of 5% tribasic sodium phosphate until the dehydrated tissue had absorbed water and presented a natural appearance. It was then washed in running tap water till the reagent had been removed. The specimen was then fixed in 10% formaldehyde and later transferred to 80% Alcohol. It remained as a spirit preserved specimen in a glass museum jar for some years. It was then washed again in running tap water to remove the alcohol and finally freeze dried. The dried hand presents an extremely lifelike appearance and without knowledge of its history would pass for a freeze dried hand obtained from a normal post-mortem human cadaver.

It may be concluded from the above examples that the technique of freeze drying is the most perfect and versatile method of preserving biological tissue yet devised. Entire animals may be processed and stored without hazard or more than routine maintenance for an indefinite period of time. Specimens are still in excellent condition from the inception of the process in 1956. Also freeze dried specimens may at any time be rehydrated for museum jar display, dissection or for microscopic examination.

Freeze drying for soft tissue stereoscan electron microscopy

Technique

Tissue to be examined, both fresh and preserved, was carefully washed in several changes of distilled water, with a final rinse in triple glass distilled water. This was necessary because it was found that normal distilled water and double distilled water left a considerable deposit of dissolved salts in the freeze dryer after sublimation. Delicate tissues were left in sufficient distilled water to support them in a life-like position. It was not necessary to support firm tissues, these simply being left in their containers with all the surplus water drained off. The containers used in this work were Durham's fermentation tubes, normally used in bacteriological technology. Each piece of tissue was treated in a separate tube. The specimen was rapidly frozen using a Polar spray, 100% dichloro-diflouro-methane in an aerosol container. The rate of freezing of this aerosol spray is comparable with CO_2 at approximately -70°C. Each small tube was taken up in a pair of forceps, sprayed for a few seconds and then placed without delay, into the specimen chamber of an Edwards High Vacuum freeze dryer. The machine had

previously been pumped down to a working pressure and temperature. These were working pressure of 50 microns Hg or 0.05 Torr., specimen chamber temperature of -15°C. and a condenser temperature of-40°C. After the samples had been sprayed and placed in the specimen chamber, the apparatus was switched on and sublimation carried out for 12 hours. After this period, the pressure was released and the specimens removed from the chamber. They were then placed into a small glass, medium vacuum, desiccator over silica gel for half an hour, to enable any condensation forming on the surface of the dried specimens after removal from the cool chamber to be removed. Specimens were then ready for stub mounting, coating and subsequent examination.

Use of liquid nitrogen for removal of debris and excess tissue

In some cases, an excess of debris or tissue was found on the samples, particularly on preserved tissues. Cleaning of the tissue was carried out, after washing and draining, using liquid nitrogen as follows. The samples, cooled for a few seconds by the Polar spray in the individual small tubes, were taken carefully in a pair of cooled Spencer-Wells forceps and dipped beneath the surface of a sample of liquid nitrogen. A small thermos flask was used as a suitable container for the liquified gas and this had been cooled for some hours in a freezer at -20°C. before attempting to pour in liquid nitrogen. It is dangerous to attempt to pour liquified gases into non-cooled glass containers. The tubes were taken from the surface of the nitrogen and placed without delay, into the specimen chamber of the freeze dryer and the apparatus switched on as for normal sublimation. The small amount of liquid nitrogen remaining in the tubes, after removal from the flask, boiled rapidly and this ebullition effectively removed debris and extra tissue not required in the preparation, from the surface of the sample, leaving clean specimens for examination.

Several hundred samples have been prepared in this way and it is now a standard procedure for cleaning tissues whenever the need arises. The sample treated with liquid nitrogen must be pre-cooled to prevent the formation of a 'Leidenfrost Envelope'. This envelope will cause considerable distortion in specimens not previously cooled. Tissues at room temperature cannot adjust to extreme temperatures successfully, without previous cooling to at least -50°C. or below. Liquid nitrogen has a temperature of -196°C. This cooling was carried out by using the aerosol spray.

Any soft biological tissue, whether fresh or preserved, may be prepared for scanning in this way. The final preparation of a clean and suitable sample may be helped by the use of liquid nitrogen.