FREEZING AND DRYING BIOLOGICAL MATERIALS

DURING the past half-century spectacular advances in theoretical and applied physics have distracted attention from biological discoveries no less fundamental, dramatic or potentially useful. For example, Spencer's theory that life involved continuous change was upset at the turn of the century by the demonstration that biochemical activity and expenditure of energy in living seeds and bacteria could be arrested for prolonged periods at very low temperatures, and were resumed together with all manifestations of normal life when they were rewarmed[1].

Gradually it became established that most animals and their component cells and tissues were killed by freezing and thawing. Some invertebrates survived desiccation at normal temperatures and intense cold in the dry state. Others, including frost-hardy insects, resisted freezing by supercooling. Experimentally, vinegar eels, frog spermatozoa and muscle fibres and mammalian erythrocytes were recovered in a viable state after minute specimens had been cooled to, and rewarmed from, very low temperatures at an ultra-rapid rate which was thought to convert the aqueous phase to and from the vitreous state and so suppress formation of ice crystals[2]. By 1948 the biological effects of very low temperatures seemed theoretically well established, and no practical applications appeared feasible.

In contrast, the process of freeze-drying was being widely used ten years ago for preserving a variety of biological products, including antibiotics, plasma, sera, cultures of living bacteria and suspensions of viruses. Great technical advances had been made since 1890 when Altmann[3] applied the method to histological fixation and since Shackell in 1909[4] preserved the biochemical components of liver slices and the pathogenicity of the rabies virus in this way. On the other hand, the theoretical basis for sublimation of ice at low temperatures *in vacuo* had changed little since Wollaston[5] demonstrated his cryophorus in 1813.

This was the background against which the Institute of Biology organized its first Symposium on Freezing and Drying in 1951[6]. On that occasion, current industrial and laboratory equipment and biological applications of freeze-drying were described, as well as preliminary research into factors involved in deterioration of products and loss of viability of micro-organisms. It was surprising to learn, at that time, that mammalian skin frozen at − 30° C. and exposed to high vacuum survived reduction of its water content from 70 to 25 per cent, and that transmission of tumours by freeze-dried material could be explained by survival of cancer cells. Two years experience had shown that, in presence of glycerol, mammalian spermatozoa, red blood cells, endocrine tissue and skin could be frozen and stored for long periods at low temperatures without loss of viability and that survival was maximal when the material was cooled comparatively slowly and rewarmed rapidly. The processes of cooling and rewarming living cells at an ultra-rapid rate to promote vitrification and vitrofusion were demonstrated by Prof. B. J. Luyet, but were clearly no longer the only or the best methods for preserving the life of mammalian cells at low temperatures.

The Second International Symposium on Freezing and Drying, organized by the Institute of Biology and supported by the Wellcome Trust, was held on April 1 and 2, 1958, in the Beveridge Hall, University of London. Dr. A. S. Parkes, in his opening remarks, mentioned the committee's hesitation to repeat a successful experiment and to test whether pooling of ideas seven years previously had, in fact, led to any advance in theory or practice.

Prof. B. J. Luyet described refinements in his techniques of microscopy and of recording temperature during ultra-rapid cooling and rewarming of thin layers of biological solutions. He showed pictures of the evanescent crystalline spherulites formed in material described as vitreous because crystals were hitherto thought to be absent. He said that the term vitrification had been used to cover over-hasty generalizations, and that fundamental facts were now emerging.

Dr. H. T. Meryman confessed that his interest in preserving cells at low temperatures dated from the previous symposium. Since then, his studies had shown that crystal formation was invariably extracellular in suspensions of mammalian cells and tissues cooled either slowly or rapidly. Minute intracellular crystal nuclei were found only in specimens cooled at an ultra-rapid rate. Damage was inevitable unless the frozen cell suspensions were rewarmed very rapidly. When small samples of frozen blood were rewarmed rapidly from low temperatures, severe hæmolysis occurred in those cooled slowly. This was not due, as previously supposed, to the mechanical action of ice crystals, but to the concentrated electrolytes formed during gradual separation of ice. Increasing the rate of cooling decreased the amount of destruction until a broad optimal range was reached. Further increase in the rate of cooling led to diminished recovery of cells after rapid thawing, resulting either from differential thermal contraction of individual cell constituents, as suggested by Lovelock[7], or else from intracellular crystallization. When blood is frozen in the presence of glycerol, rates of cooling and rewarming are less critical because glycerol molecules bind water molecules which reduce the concentration of electrolytes during freezing and thawing. Large samples of glycerol-free blood in containers of certain shapes can be cooled at an optimum rate throughout, but thawing at an ultra-rapid rate throughout is more difficult and may be impossible.

Hitherto it has been assumed that bacteria, yeasts and moulds survived freezing whatever the rate of temperature change. Dr. P. Mazur has now shown that *Pasteurella tularensis*, *Saccharomyces cerevisiae* and spores of *Aspergillus flavus* are severely damaged when rewarmed slowly after freezing at temperatures below a critical level characteristic for each species, generally between − 10° and − 45° C. The proportion of viable cells is further reduced in rapidly cooled samples. When the specimens are rapidly rewarmed the majority survive regardless of the rate of cooling and minimum temperature. A high proportion of cells must have been potentially viable when frozen and were killed during rewarming. Very rapid cooling may cause intracellular freezing which renders the cells susceptible to damage during slow

© 1958 Nature Publishing Group

warming. Prof. L. R. Rey's microscopic studies show that conversion of minute intracellular crystals to large ones may disrupt cells cooled rapidly to low temperatures (particularly if they are then kept at temperatures above − 40° C.) and during slow thawing. When the electrical conductivity of physiological salt solutions is plotted against temperature the shapes of the respective curves during freezing and thawing differ, whereas in presence of glycerol they are identical and the eutectic point is lowered to − 60° C. When tissues or biological media are kept at temperatures between − 60° and − 190° C. striking thermodynamic changes occur, particularly in the presence of glycerol and at the higher temperatures. Relative stability is reached at the temperature of liquid nitrogen (− 196° C.). The loss of viability during prolonged storage of certain tissues in saline media containing glycerol at the temperature of solid carbon dioxide (− 79° C.) may be connected with the changes in physical state of ice reflected by these thermodynamic effects.

Prof. N. Kalabukhov's paper, read in his absence, reviewed Soviet work of the past sixty years on problems of cooling whole animals to sub-zero temperatures. Poikilotherms survive superficial freezing, but neither poikilotherms nor homeotherms recover after freezing the whole organism. There is little prospect of preserving whole animals by vitrification. By contrast, some invertebrates and poikilothermic vertebrates recover after prolonged periods of supercooling. Supercooled bats have also survived storage for several days at − 5° to − 7° C. Heart beats were recorded on electrocardiograms, showing that living processes were not completely suppressed. Recent methods for re-animating mammals after severe hypothermia and cardiac arrest re-open the possibility of long-term storage of a wide variety of whole animals at sub-zero temperatures in the supercooled state[8].

Since 1951 mathematical problems of freeze-drying have been analysed. Dr. J. L. Stephenson has determined the probability that a water molecule which escapes from the ice surface will not return, but will be permanently removed from the system. This factor depends on the intrinsic structure of the material being dried and determines the design of the apparatus. Deterioration of freeze-dried products during storage is known to be related to final moisture content, which may be 7 per cent of the dry weight after sublimation of ice. It is further reduced by a process of secondary drying, the physics of which was explained by Dr. T. W. Rowe and Mrs. E. Robson. The all-important question is: How much moisture should be left in a product? This raises the controversial subject of 'free' and 'bound' water molecules and the technical difficulties of determining moisture content.

The residual moisture-content of different materials depends upon their respective hygroscopicities and upon the vapour pressure of the atmosphere in equilibrium with them. Dr. W. J. Scott has freeze-dried samples of bacterial cultures in different media and has stored them, either in air or *in vacuo*, in hermetically sealed containers over solutions which produce a known vapour pressure. The optimum moisture-content for long-term storage of freeze-dried bacteria depended upon the presence or absence of air, and upon the composition of the original suspending fluid. Dr. Scott's results support the theory that death during storage is due to 'browning reactions' between carbonyl groups in the medium and amino groups in the micro-organisms. Survival is improved by replacing glucose, fructose or ribose by the corresponding reduction compounds, sorbitol, mannitol or ribitol, or else by sucrose, which lack carbonyl groups. Alternatively, carbonyl groups in the medium can be destroyed by reagents such as hydroxylamine. Addition of amino-acids, which compete for carbonyl groups, is equally effective.

Following these principles, Dr. P. W. Muggleton has achieved a high constant survival-rate of attenuated tubercle bacilli in B.C.G. vaccines stored for prolonged periods after freeze-drying. His medium contains 1–2 per cent sodium glutamate, 8 per cent dextran and 7·5 per cent sucrose. Survival is maximal when primary drying is carried out at − 25° to − 28° C. and when the cultures are sealed *in vacuo* after secondary drying to a residual moisture content between 0·8 and 1·0 per cent. Dr. R. I. N. Greaves assesses the effect of the medium on subsequent stability by boiling the freeze-dried cultures. Paracolon bacilli and gonococci dried in presence of 5 per cent sodium glutamate and 5 per cent dextran survived boiling in maximal numbers. Several of those present questioned whether viability after boiling represents potential viability of a dried culture during prolonged storage.

Freeze-drying is used by Miss B. L. Brady as an ancillary method of storing yeasts in the National Collection of Type Cultures. Its exclusive use would economize time and materials, but would be hazardous until the complex factors influencing the viability of yeasts of different genera are better understood. Rehydration of the desiccated cells is probably the most critical procedure. So far, she has observed no qualitative changes in nutritional requirements or biochemical reactions of yeasts preserved by freeze-drying.

To-day, freeze-dried rinderpest-virus vaccines are used on a large scale for veterinary prophylaxis. Dr. G. R. Scott and Dr. C. S. Rampton described difficulties which have been surmounted in Kenya in preparing large batches of vaccine from inoculated rabbits and goats. Dr. D. Greiff finds that the infectivity titre of influenza viruses is not significantly altered by freezing slowly and drying by vacuum sublimation at − 80° C., but is reduced by storage at temperatures above −40° C. or by drying at temperatures above −80° C. Studies on frozen mitochondria show that uptake of oxygen is little affected by the rate of cooling, but that phosphorylation is greater in suspensions cooled slowly to − 40°, − 63° or − 192° C. than in those frozen rapidly at the same temperatures. Lesions to mitochondria might be a cause of death in intact cells. In cells disintegrated by freezing and thawing, the apparent numbers of cellular or infective virus particles and the resulting enzymic activity will depend on the minimum temperature and rate of change of temperature.

Banking frozen and freeze-dried human tissues for future surgical use is a new development since 1951. Commander G. W. Hyatt described the organization of the tissue bank at the U.S. Naval Medical Center, and the techniques used there. Skin, arteries, cartilage, bone and other tissues are removed from fresh cadavers of healthy young adults and are frozen or freeze-dried and afterwards homografted in suitable cases. The clinical results leave no doubt of the efficiency of the thawed or reconstituted grafts, although the constituent cells are dead. The implanted tissue acts mechanically, providing an

©1958 Nature Publishing Group

anatomical template for ingrowth of the host's tissues. The process of freeze-drying apparently alters the nucleic acids in the homografts, reducing their capacity to evoke immunological responses. Commander Hyatt also discussed the ethics and philosophy underlying this enterprise.

Prof. C. G. Rob said that in his clinical experience the general health of the patient and state of his other arteries are the most important factors governing a successful outcome of homografting frozen or freeze-dried arteries. Mr. H. A. Sissons finds that freeze-dried bone sterilized by irradiation has advantages over homografts preserved by other means. Grafting the cornea presents unique possibilities and problems. Because the tissue is avascular, immunological reactions to homografts do not occur, but viability was apparently essential if grafts were to retain transparency. Col. F. Hénaff has, however, obtained striking results with freeze-dried corneal homo- and hetero-grafts in rabbit, dog and man. Lamellar grafts invariably took, and some of the full thickness homografts also remained transparent although the tissue was undoubtedly dead.

By contrast, pathogenic protozoa, spermatozoa and endocrine tissue can never perform their specific functions unless they are alive. They can be preserved for long periods in the frozen state in presence of glycerol at low temperatures, but cannot be stored after freeze-drying from this medium because of the lethal effects of concentrated glycerol at room temperature. Dr. C. Polge and Dr. M. A. Soltys reported that, in presence of 7·5–10 per cent of xylose, glucose or sucrose, and in absence of salts, bull spermatozoa suspended in egg yolk and trypanosomes in 10 per cent horse serum survived freezing at temperatures down to −40° C. In these media they might survive freeze-drying. Dr. Parkes envisaged the protection of endocrine tissues during freezing by substances which would volatilize together with ice during high-vacuum distillation. Methanol is sufficiently volatile at low temperatures, and rat ovaries have survived freezing for several hours in its presence. Future developments along these lines may eventually permit long-term storage of mammalian cells without loss of viability in the dry state at room temperatures.

The interest of the meetings was unquestionable and the proceedings, to be edited by Dr. A. S. Parkes and published by Blackwell Scientific Publications, Ltd., should provide a basis for further advances in this branch of natural philosophy. The secretary of the Symposium, Dr. R. J. C. Harris, and the Institute of Biology, are again to be congratulated on their initiative.

AUDREY U. SMITH

[1] Smith, A. U., in "Biological Applications of Freezing and Drying", ed. by R. J. C. Harris (Academic Press, Inc., New York, 1954); *Biol. Rev.*, **33**, 197 (1958).
[2] Luyet, B. J., and Gehenio, P. M., "Life and Death at Low Temperatures" (Biodynamica, Normandy, Miss., 1940); in "Temperature: Its Measurement and Control in Science and Industry" (Reinhold Publishing Corp., New York, 1941); *Colloid Chem.*, **5**, 859 (1944).
[3] Altmann, R., "Die Elementarorganismen und ihre Beziehungen zu den Zellen" (Veit and Co., Leipzig, 1890).
[4] Shackell, L. F., *Amer. J. Physiol.*, **24**, 325 (1909).
[5] Wollaston, W. H., *Phil. Trans. Roy. Soc.*, **103**, 71 (1813).
[6] Harris, R. J. C., "Freezing and Drying" (Institute of Biology, London, 1951); *Nature*, **168**, 851 (1951).
[7] Lovelock, J. E., *Nature*, **173**, 659 (1954).
[8] Andjus, R. K., and Smith, A. U., *J. Physiol.*, **128**, 446 (1955); Smith, A. U., Lovelock, J. E., and Parkes, A. S., *Nature*, **173**, 1136 (1954).

NOISE IN INDUSTRY

NOISE in industry was the subject of the eighth conference of the British Occupational Hygiene Society, which was held in London on April 1.

The proceedings opened with a paper on the clinical and physiological effects of noise by Mr. T. Cawthorne (King's College Hospital, London), who said that hearing was the last of the special senses developed in animals in the course of evolution. The reception apparatus arose as an outgrowth of the organ of balance, and disorders of hearing were accompanied by disorders of balance. Hearing was primarily a protective device which operated through the central connexions of the eighth nerve, some of which went to the temporal lobe, where sound was appreciated, and some directly to motor nerves to produce a sound reflex action even before the animal was conscious of the sound it reacted to. Propagation of the species was another function which was often aided by hearing, in man as well as in the lower orders, but man was unique in his possession of speech and in his social activities, which depended very much upon unimpaired hearing. Deaf persons were under a constant stress on this account.

Sound waves in air passed down the ear canal and generated vibrations of the ear drum which were transmitted across the air-filled middle ear by a chain of ossicles. The innermost of the ossicles rocked in the oval window of the inner ear, or cochlea, and caused oscillations to pass through the fluid which filled it. The fluid waves caused the basilar membrane of the cochlea to spring up and down. This membrane carried the organ of Corti, which converted the movements to tactile stimuli of nerves. The organ carried hair cells which were brought into contact with a leaf-like structure above them when the basilar membrane moved. Excessive movement strained the hair cells and acoustic trauma took place mainly, if not entirely, in the organ of Corti.

Early deafness resulting from exposure to noise very often produced a loss in sensitivity to frequencies in the vicinity of 4,000 cycles. This was found in men employed in metal stamping and chipping, and in those who shot two or three thousand cartridges a year.

Both the intensity of noise and its duration were significant in causing trauma. Regular exposure over long periods to more than 85–90 decibels resulted in irreversible changes taking place in the organ of hearing. Individual sensitivity varied and unduly sensitive entrants to industry should be picked out by tests. One way of doing this was the estimation of post-stimulatory fatigue.

High-tone deafness made it difficult to interpret speech, particularly when there was a noisy background; good binaural hearing was an asset in the latter situation. High-tone deafness caused by industrial trauma resulted in a time lag becoming apparent in the apprehension of speech. The importance of speaking slowly when addressing an audience arises from this effect, which might also be due to fatigue on the part of the listeners.

The encouragement of workers to protect themselves against excessive noise called for propaganda. There was little hesitation in guarding against an obvious

©1958 Nature Publishing Group