PART III

Production operations

Basic laboratory services and media preparation for vaccine production

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The services to a vaccine production unit should be of the highest standard attainable and should provide a structure that is appropriate, taking into consideration the conditions and resources normally expected in a developing country. The factors that have a fundamental influence on the quality and sustainability of these services are:

- *Water supply.* Methods of water treatment should ensure high quality, and the most appropriate equipment to achieve a high standard of final water quality should be selected.
- *Electricity supply.* This service is vulnerable to supply interruption and its quality is often beyond the control of the end-user. Protective devices should be installed to ensure an uninterrupted, stabilized power supply, independent of the generating authority. Although the equipment used in the central services section is not as vulnerable to voltage fluctuation and supply interruption as the more sensitive equipment found in production units, it still requires a constant and uninterrupted supply to maintain a high standard of service.
- *Capital equipment.* Careful consideration must be given to the selection of the equipment needed to operate a central services section successfully. It should always be borne in mind that the most sophisticated is not necessarily the best and the natural desire to incorporate the latest technology should be carefully evaluated before a decision is made. At the early planning stages, scale is also a very important factor to consider.

WATER

Water is undoubtedly the most vital and fundamentally important resource required by the central services and media departments. It is therefore extremely important to ensure its uninterrupted supply and quality.

The quality of the raw water available to a laboratory depends to a great extent on the geological stucture of the catchment area and the nature and quantity of the minerals dissolved in it. In many cases the laboratory has no control over the selection of source and is obliged to rely on the supply provided by the local water authority. The source can be a well, a spring or a borehole, adjacent to and under the control of the end-user. However, the water quality is still dependent on the geological strata surrounding the aquifer.

The supply of clean drinkable water in developing countries varies considerably. In many cases the supplying authority will have carried out some form of pretreatment to give a supply fit for human consumption. Generally this means chemical flocculation followed by filtration and chlorination. These processes can introduce an increased chemical composition which must be removed before the water can be used for vaccine production. In many arid catchment areas, boreholes and wells that suffer seasonal variation are often contaminated further

with particulate matter in the form of colloidal clays and laterites and may not be subjected to treatment by the local water supply authority. In such cases, the laboratory should pretreat the waters before attempting further purification.

Methods of determining the quality of the raw water

Optical clarity. This is usually obvious and can easily be determined by visual observation of a sample in a glass beaker against a transmitted light source.

Soluble mineral salts and ionic composition. A reliable measurement of the degree of dissolved solids in raw water can be made using a conductivity meter which measures its electrical conductivity in microsiemens per centimetre. This will give a good indication of the purity of raw water. Waters with a high mineral content can give readings as high as 1 500 microsiemens/cm whereas softer waters with lower mineral content can be in the region of 50 to 200 microsiemens/cm.

It should be emphasized that this measurement on its own, although a valuable indication of the general purity, cannot identify the soluble salts of calcium and magnesium which, in the main, are the cause of damaging permanent hardness. A simple colorimetric test kit is available which will determine the relative degree of hardness in the water sample and thereby indicate if the water is hard, medium or soft.

In general, soft waters, if not contaminated with particulate matter, are perfectly suitable for all washing operations and for direct feed to stills and deionizers without pretreatnent. If particulate matter is found to be a problem it can be removed effectively by coarse-clarifying unglazed porcelain candle filters which can be incorporated between the equipment and the raw water supply.

This type of filter is recommended for the small vaccine production unit because it presents a sustainable type of system, will give years of trouble-free operation and does not rely on the frequent importation of disposable cartridges which some current systems require. Furthermore, these filters can be dissassembled easily by unskilled operators, scrubbed by hand, backwashed to remove the surface film of particulate material and be back in operation within a short time.

Equipment for water purification

At this stage some consideration has to be given to scale and the volumes of media and vaccines to be produced as these have a direct bearing on the size and output of the equipment to be installed. In large units with high consumption, where raw water is pumped from a borehole or drawn directly from a river or stream and is heavily contaminated with particulate and colloidal matter, it might be advisable to treat all raw water entering the service unit by installing a sand filtration system to reduce suspended solids to less than 40 microns and give flow rates of 50 litres per minute. Such equipment is available commercially and is simple to operate and maintain, no chemical regeneration is necessary and it requires only a physical backwash service regeneration operated by a seven-day timer. For vaccine production units producing a total of approximately 15 000 litres of mixed viral and bacterial vaccines per annum, semi-industrial scale water purification equipment is the best option. It is wiser to install appropriately sized equipment which will be fully utilized rather than opting for prestigious large-scale equipment which may be grossly underutilized and involves high capital expenditure. Future increased demand should be anticipated, however, and the capacity should be able to cope with at least a doubling of demand.

Water-softening plant

Medium- to large-scale vaccine producing units in developing countries frequently opt for a central semi-industrial water-softening plant which treats all incoming raw water before reticulation to the various sections for individual uses. Although this may seem to be a good practice, such a system continues to operate effectively. Without strict monitoring and the delegation of responsibility to a responsible operator, the system breaks down and generally falls into disuse. It is also wasteful to soften all incoming water when much of it will be used for general purposes for which softened water is unnecessary and when softening large quanitites of water requires frequent regeneration of the equipment which results in operator fatigue and neglect. It is often better to install smaller individual softeners at the points where they are most required, such as the bottle cleaning department and, where necessary, to protect stills and electrically operated steam generators.

The decision to install water-softening plant is, therefore, entirely dependent on the degree of hardness of the raw water. In general, water with a degree of hardness greater than 10°dH should be softened for laboratory use, and many natural raw waters have values much greater than this (e.g. 28° hardness). A plant that delivers approximately 110 m^3 of water at 1° hardness between regeneration cycles will be adequate for most small- to medium-scale vaccine production units.

Water-softening plants are usually of the single bed resin ion exchange type, regenerated with sodium chloride. Modern units are completely automatic and have a monitoring device which actuates the regeneration cycle when the degree of hardness becomes unacceptable. The operator has only to maintain a suitable level of salt in the regeneration reservoir. Uses for the softened water include the first wash in the bottle cleaning section, where it can greatly reduce the need for expensive detergents and soaps, and for the protection of stills, where it eliminates the build-up of damaging calcium and magnesium scale on the heating elements. Softened water should not be used to protect deionizers as the effluent contains a high concentration of sodium ions which would rapidly exhaust the ion exchange resins of the deionizer.

Water pressure

Water pressure is an important consideration and for the efficient operation of water softeners, deionizers and automatic stills the pressure on the equipment at raw water inlets should be at least 2.5 bar.

Deionizers

Deionizers are very useful for the culture media and bottle cleaning departments of the vaccine production unit where large volumes of relatively pure water are required. Most modern equipment is capable of delivering high-quality water with a purity matching glass double-distilled water of around 0.08 microsiemens/cm.

A moderately sized twin bed or mixed bed system will provide water at a flow rate of 300 litres per hour for the final rinsing of glassware and for all culture media preparation with perhaps the exception of tissue culture media which ideally should be depyrogenated.

Water purification systems based on ion exchange, reverse osmosis or a combination of both are available for the laboratory and vaccine production unit. Most systems rely on expensive factory-produced cartridge replacements when the resins are exhausted. This system is very difficult to sustain in a developing country where extreme demands on hard currency

reserves lead to extended use of exhausted cartridges and a resulting fall in water quality.

A more sustainable system is one which utilizes automatic electrical flushing regeneration of the resins *in situ* whereby the resins do not require chemical regeneration nor do they become exhausted and need expensive replacement. Flow rates of 1 200 litres per hour and greater are possible with equipment only 1 m high and occupying less than 0.3 m^2 of floor space. If the equipment does not have a built-in conductivity meter, monitoring the conductivity of the effluent from the deionizer can be done by using a hand held meter.

Stills

With the exception of the most sophisticated deionizers, which contain depyrogenating cartridges, the all-glass double-distillation still is undoubtably the most reliable and sustainable system for the production of pyrogen-free water with a conductivity lower than 0.1 microsiemens/cm suitable for cell culture.

Modern automatic stills are compact and very reliable pieces of equipment which will give years of trouble-free service as long as they are protected from scale formation by the upstream installation of a suitable water softener when used in hard water areas.

Most modern equipment is provided with fail-safe and automatic cleaning devices which make it very easy to operate. Glass double-distillation units are available with outputs of up to 8.0 litres per hour and, with suitable upstream protection and a reliable power and water supply, these can be operated continuously providing ample water for cell culture vaccines.

ELECTRICITY

The central services unit must be connected to an automatic trip standby generator in order to ensure a completely uninterrupted electricity supply. Each section should be provided with sufficient power outlet sockets rated at 15 amps connected to a sub-distribution board containing automatic trip miniature circuit-breakers to protect all equipment from overloading and short-circuiting. The sockets should be located 15 cm above bench height and must be properly earthed. Sockets in the media preparation and bottle cleaning sections should ideally be of the splash-proof type.

Where three-phase 380/400v supply is required for larger equipment such as autoclaves, stills and steam generators lines should terminate via a suitable automatic trip fused switch-box.

Voltage stabilization is not normally required in the central services section as most of the equipment there is fairly robust. With the increased use of more sophisticated, microprocessor-controlled systems, however, it would be advisable, if the vaccine production laboratory is already served with a stabilized supply, to install an outlet in the media preparation room. The cold room and freezer room should be connected to a dedicated electrical supply, i.e. each piece of equipment should have its own line which is not connected to any other equipment, to avoid loss of function owing to accidental overloading with subsequent fuse trip disconnection.

Main and sub distribution boards should be equipped with an electromechanical interconnection that effectively disconnects all power when the protective cover is opened for inspection.

Electrically operated equipment handled in wet areas such as the media preparation section should be provided with residual current detection devices that switch off the supply to the equipment to protect the operator from possible electrocution in the event of serious current leakage.

THE CENTRAL SERVICE UNIT: DESIGN AND OPERATION

A central services unit is generally composed of four distinct sections:

- the decontamination section;
- the bottle cleaning section;
- the media preparation section;
- the sterilization section.

Each section has its own special function which is distinctly related and physically linked to those of the other sections in such a way as to provide the most efficient use of space and to adhere to the rules of good manufacturing practice (GMP). Ideally the central services unit should be located on the same floor as the vaccine production section and both should be accessible by a shared service corridor. In designing a new central services unit or modifying an existing one, careful attention should be given to the traffic patterns of both personnel and materials within the related sections to ensure efficient and safe operation.

The unit can be constructed from hollow concrete blocks or bricks faced with a high-quality cement screed and finished with waterproof plastic paint. All electrical wiring should preferably be surface-mounted using a ring main layout from a main distribution board through suitably fused miniature circuit-breakers to surface mounted outlet sockets located 15 cm above bench height. Where three-phase supply is required for stills, electrically heated autoclaves, steam generators and hot air ovens the outlets should be terminated with appropriately fused isolator switch-boxes. In wet areas such as the bottle cleaning and media preparation rooms all outlet sockets should be of the splash-proof type and suitable earthing should be mandatory. Great care must be taken to ensure that there are sufficient outlet sockets in each section to avoid the common and dangerous practice of overloading outlets with multiple adaptors.

The floors throughout the central services section should be of high-quality waterproof terrazzo continued 15 cm up the walls and, with the exception of those in the sterile filtration and hot and cold rooms, all floors should be provided with adequate falls to a common glazed earthenware or plastic drain running the full length of the outside walls and covered with a removable, durable, perforated grating. This last feature is essential, especially in the media preparation section, to facilitate the washing and chemical decontamination of the high-traffic working areas.

The use of factory-prepared, pre-fabricated benching and "flat-pack" furniture made from melamine-surfaced high-density particle board is a cheap and practical solution. This furniture can be installed by relatively inexperienced staff and is very flexible in form and function.

The decontamination section

The decontamination section is designed to deal only with hazardous contaminated materials originating from the vaccine production unit. It should be accessible from all sections of the vaccine production unit and be completely self-contained with a single access from the shared service corridor.

This section should contain a suitable reception table on which the material to be decontaminated is placed. Ideally the contaminated material should be sterilized in a double-ended autoclave, the dirty side of which is accessible from the decontamination section and the clean side from the adjacent room which houses the bottle cleaning section. All material entering the autoclave in the decontamination section should be identified with autoclave tape which will give the operator receiving it in the bottle cleaning area clear visual evidence that the load has been sterilized.

Autoclaves of this type are generally

operated by mains steam originating from either a central boiler house in relatively large vaccine production units or from smaller electrically operated steam generating units. Steam pressure of at least 15 kg per square centimetre is required to operate this type of autoclave, which should also be fitted with a water-operated vacuum system to remove trapped air and ensure efficient penetration of the load by superheated steam. The autoclave should be fitted with safety door locks to prevent the opening of either door until the pressure and temperature are within safe operational limits and present no hazard to the operator.

The cycle should be electronically controlled by solid-state circuitry. A truck and rail system for loading and unloading should be incorporated to permit complete removal of the load before handling.

Bottle cleaning

The bottle cleaning section should be situated adjacent to the decontamination section and is used for the cleaning of all glassware and equipment originating from the decontamination autoclave and of uninfected material from the vaccine unit. It houses the deionizer, still and water softener. It should have a reception bench on which all dirty but uninfected glassware, etc. can be placed. This material, together with the decontaminated contents of the double-ended autoclave, is then subjected to the washing / cleaning process.

The bottle cleaning section should have three large deep polypropylene sinks fitted with large-bore vertical overflow pipes and supplied with hot and cold water. The local construction of concrete, terrazzo or tiled sinks is not recommended since, invariably, such sinks lead to unacceptable glassware breakages and are difficult to keep clean.

It is important to consider the work flow pattern. Most operators are right-handed, so it is covenient to plan an anticlockwise flow pattern. Sterilized previously infected material originating from the double-ended autoclave and uninfected dirty material should be collected on a large table to the right of the operator. The glassware is then subjected to a hot water and detergent soak in the first sink, followed by brushing, if necessary, to remove stubborn adhesions with an electrically powered bottle brushing machine. The glassware is then transferred to the second sink on the operator's left, where it is rinsed with hot water. In the third sink the glassware receives a final rinse in deionized water and is left to drain on racks. The clean, dry glassware can then be stored in a room designated for non-sterile clean glassware in this section.

The rinsing of glassware is best carried out using pressure jet nozzles located at the base of the tap-water and deionized water rinsing sinks. These nozzles can be adapted to suit all types of bottles, test tubes, etc. and are available commercially from laboratory equipment suppliers. For larger vaccine production units it may be desirable to consider a fully automatic bottle washing machine but in most developing countries it is probably more practical to utilize human operators using the simpler equipment described previously.

Media preparation section

The media preparation section requires very careful consideration and the ultimate layout and size will depend largely on the batch sizes that are to be produced. If production batch volumes are to be greater than 50 litres per batch, it will be necessary to consider the installation of equipment capable of handling larger volumes – up to 100 to 200 litres per batch. In such cases, it is better to install steam-heated hemispherical stainless steel vessels complete with rim-mounted electric

stirrers. The most convenient and practical layout would be one 50-litre capacity stainless steel hemispherical vessel fitted with a mild steel jacket and mounted on four mild steel legs and two 200-litre vessels of a similar design. The steam jacket of each vessel should also have a cold water supply to facilitate rapid cooling when required.

These three vessels constitute the preparation vessels necessary for culture media prepared from dehydrated ingredients and for the preparation of protein hydrolysates. They should be situated adjacent to one another and all steam trap and waste water outlets should be connected to a suitable floor drain along an exterior wall. All the steam pans should be covered by a suitable extraction hood venting through an adjacent exterior wall. The floor of the media preparation room should be constructed of high-quality terrazzo with a suitable fall to a glazed earthenware or plastic floor drain, covered with a removable perforated cast iron or alloy grating. The terrazzo flooring should be continued 15 cm up the walls to complete a well-drained, durable, washable surface. Culture media prepared in the vessels described above can be transferred directly by peristaltic pumps through high-capacity membrane filters housed in an adjacent sterile filtration room. The sterile filtration room should be equipped with a horizontal laminar flow cabinet and a stainless steel sink supplied with cold water and two bench-mounted gas taps connected by copper tubing to a cylinder of propane located in a well-ventilated gas store outside the building. The sterile filtrate can either be pumped directly into sterile fermenters housed in the adjacent vaccine production unit or be delivered into the appropriate final sterile glass or disposable plastic growth bottles in the filtration room prior to transfer to the production unit.

All operations within the sterile filtration room should be carried out using aseptic precautions.

Large-scale culture media which is to be sterilized *in situ* can also be pumped directly into fermenters or suitable containers using peristaltic flow inducers. This type of pump is highly recommended as the simplest means of handling liquid for both direct transfer and positive pressure filtration.

In-house processing of culture media raw materials. In vaccine production units with a suitable supporting infrastructure, where large volumes of culture media are used and where local resources such as mammalian protein in the form of waste (condemned) meat, milk by-products such as whey, vegetable protein by-products such as maize gluten and waste blood from abbatoirs are available, the processing of such raw materials can be considered as a means of increasing self-sufficiency and reducing dependency on costly imported dehydrated media.

Hydrolysates of these materials can be prepared using the three vessels already described, concentrated by vacuum evaporation to a density of 40 percent total solids and stored at -20°C or, preferably, spray-dried to give a free flowing powder of greater stability that does not require refrigerated storage.

The media preparation section should have access to walk-in refrigerated rooms at -20°C and 4°C for the storage of perishable raw materials and finished sterile media and walk-in incubators at 37°C for the sterility testing of filtered media in bulk containers.

A complete set of formulae and standard operating procedures should be available for all culture media prepared in the section and copies distributed to all end-users. A review procedure should be adopted where new formulae can be included and

existing ones upgraded when necessary. A customer request system should be instigated to help ensure the smooth operation and execution of orders and to help maintain a record of raw material consumption.

A batch numbering system should be operated for all batches of media prepared to enable identification in the event of end-user complaint and to help identify any associated procedure that may have had either a beneficial or a deleterious effect. Following sterilization by autoclaving or filtration, a representative sample of all batches of culture media should be subjected to a period of incubation at 37°C for not less than 21 days as a check on the efficacy of sterilization procedures. When large volumes of a medium are filtered without the inclusion of antibiotics, for example volumes of 20 litres in glass vessels to supply a continuous culture fermenter, it is essential to ensure the sterility of the medium by incubating the whole batch prior to use, thus avoiding costly contamination and the subsequent interruption of production schedules. The incubation period should be long enough to reveal contamination without diminishing the nutritive properties of the medium.

Sterilization section

Situated adjacent to and linked by a door into the media preparation section, this room should house the sterile autoclave and hot air oven. It is used for the final sterilization, by autoclaving or dry heat, of glassware, media and other materials from the vaccine production sections. The sterilization section should be equipped with at least two four-wheeled trolleys for the transportation of material to the vaccine production unit.

All material leaving this section should be clearly identified as sterile by the use of heat-sensitive autoclave tape secured to each item and showing unequivocally that it has been exposed to sterilizing temperatures for the necessary period.

Equipment for the central services section

Decontamination section. This section should be equipped with:

- a double-ended autoclave operated by mains steam with interior dimensions of 120 cm wide, 150 cm high and 250 cm long;
- a vacuum steam extraction facility;
- free steaming and pressure cycles that are electronically controlled;
- thermal safety locks on all doors;
- a steam-jacketed load-drying facility;
- wheeled trucks and rails for easy loading and unloading.

Bottle cleaning section. This section should be equipped with:

- a floor-mounted continuous deionizer that is electrically regenerable *in situ* and has a capacity of 1 200 litres per hour at 1.0 microsiemen/cm (e.g. Millipore DI system Model No. 9042);
- a floor-mounted water softener that is regenerable *in situ* using sodium chloride (NaCl) and has a capacity of 1 500 m^3 at 1°dH;
- a wall-mounted all glass double-distillation unit with automatic cleaning and sterilizing cycles, power and water fail-safe protection and a capacity of 8.0 litres per hour at less than 1.0 microsiemen/cm, pyrogen-free distillate (6.5 kilowatt, three-phase 380/400 V, 50 Hz);
- an electrically operated bottle brushing machine complete with assorted bottle brushes (220 V, 50 Hz);
- two heavy-duty free-standing, pressure-operated bottle/test tube rinsers for raw water and deionized water rinsing;
- a peristaltic flow inducer with variable speed control and a pumping rate from

140 ml to 33 litres per minute (220 V, 50 Hz, e.g. Watson Marlow 701U/R);
- three heavy-duty black polypropylene sinks with wide-bore vertical overflow pipes and traps, 100 cm long, 50 cm wide and 40 cm deep;
- a wall-mounted draining rack;
- a floor-mounted hot-air drying oven with fan blower, variable temperature adjustment (50° to 200°C) and timer operation, internal dimensions of 100 cm wide, 200 cm high and 150 cm deep, with adjustable shelving;
- a hand held conductivity meter with digital readout multirange of 0.1 to 2 000 microseimens/cm;
- four 100-litre polypropylene aspirators with taps, mounted on fully castoring wheeled bases.

Media preparation section. This section should be equipped with:
- two stainless steel, 200-litre capacity, steam heated, mild steel jacketed, hemispherical bottomed boiling vessels mounted on four mild steel tubular legs, fitted with steam/water vapour traps and cooling water entry and exit to the mild steel jacket and with rim-mounted 0.5 horsepower (about 375 watts) stainless steel stirrer;
- a stainless steel, 50-litre capacity, steam heated, mild steel jacketed, hemispherical bottomed boiling vessel mounted on four mild steel tubular legs, fitted with steam/water vapour traps and cooling water entry and exit to the mild steel jacket and with rim-mounted 300-watt stirrer.
- two graduated stainless steel jugs of 2-litre capacity;
- two hemispherical scoops of 2.5-litre capacity;
- a stainless steel heavy-duty dial-type centigrade thermometer;
- a stainless steel plate (40 cm by 40 cm) and frame filter press containing 20 plates with transfer plate, fitted with Saunders inlet and outlet valves with 30-mm rifled nozzles, mounted horizontally on a wheeled chassis;
- a heavy-duty peristaltic flow inducer with capacity of 140 ml to 33 litres per minute (e.g. Watson Marlow 701U/R);
- a peristaltic flow inducer/dispenser for the calibrated delivery of sterile culture media, dose range of 1.0 ml to 16 litres with positive cut-off control and with a 20:1 control ratio (e.g. Watson Marlow 501Z/R);
- a stainless steel 293-mm diameter membrane filter using 0.22-micron filtration discs (e.g. Millipore or Sartorius);
- ten heat-sterilizable polysulphone 100-mm diameter pressure-type filters using membrane 0.22-micron micro-pore filters;
- a multidisc cartridge filter using 0.22-micron PVDF filters with a maximum flow rate of 16 litres per hour;
- two hot plate magnetic stirrers with assorted polytetrafluoroethylene-(PTFE)-covered followers (1 000 watt, 220/240 V, 50 Hz);
- a bench model pH meter with five spare electrodes;
- two hand held battery-operated pH meters with ten spare batteries and five electrodes;
- a water bath in stainless steel, 50 cm long, 30 cm wide and 30 cm deep, with gabled lid;
- a heavy-duty bench-mounted butcher's meat mincer with feed-hopper, spare cutting blades and discs;
- six seamless stainless steel, 100-litre capacity containers with lids, mounted on fully castoring detachable bases;
- a laminar horizontal flow product protection cabinet;
- four stainless steel funnels, 50-cm diameter;

- two stainless steel powder scoops, 1-kg capacity;
- an electronic top-loading balance with 5 000 g capacity and sensitivity/readability of 1.0 g;
- an electronic top-loading balance with weighing range of 0 to 200 g and readability of 0.0001 g;
- an electronic platform scale with weighing range of 0 to 50 kg and readability of 100 g;
- an electrically operated steam generator providing dry steam at 15 kg/cm^2, with total working capacity to provide steam for two autoclaves and three steam-heated boiling vessels in the media preparation room.

Ancillary equipment for dehydrated media production. The media prepartion section will also require the following equipment if dehydrated media are to be produced:

- a steam-operated falling film evaporator for the concentration of dehydrated media preparations and protein hydrolysates;
- an electrically heated spray-dryer for the drying of dehydrated media and hydrolysed protein concentrates;
- six polypropylene measuring cylinders of 2-litres volume;
- six polypropylene measuring cylinders of 1-litre volume;
- six polypropylene measuring cylinders of 500-ml volume;
- six pyrex glass beakers of 2-litres volume;
- six pyrex glass beakers of 1-litre volume;
- six pyrex glass beakers of 500-ml volume.

Sterilization section. This section should be equipped with:

- a single-doored mains steam-operated autoclave with water-operated steam evacuation system, steam jacketed for load-drying, free steaming and pressure cycles operated by electronic circuitry, thermal safety locks on door, operation cycle chart recorder, wheeled truck and rail loading system;
- a floor-mounted hot air oven with blower fan, temperature control of 50° to 300°C, timer-operated cycle, stainless steel lined with multiposition shelving, 100 cm wide, 200 cm high and 150 cm deep;
- two double-shelved, fully castoring four-wheeled rubber-tyred trolleys.

Laboratory furniture. Laboratories have the following requirements:

- laboratory bench tops made from 40-mm thick, high- density particle board (680 kg/m^3), with the upper surface covered in factory-fabricated melamine, heat- and chemical-resistant plastic with one long edge post formed, 3 000 mm by 600 mm by 40 mm;
- T-profile white plastic or aluminium jointing strips to conceal joints in worktops;
- silicone rubber sink sealant for the water-repellent sealing of sinks and joints in worktops.
- five under-bench floor units acting as worktop supports constructed from 18-mm high-stability melamine-faced high-density particle board, assembled by means of integral rapid assembly slotted turn catches, with cupbboards supported on black plastic adjustable legs, doors hung on 170° self-closing hinges, fitted with flush full-width aluminium finger pulls and drawers constructed from 12-mm plywood with runners of epoxy-coated steel on nylon roller carriages. The floor units should be of the following designs: i) two cupboards and two drawers; ii) four drawers; iii) single cupboard; iv) three drawers; and v) single cupboard and single drawer;

- a sink unit cupboard;
- three single-drainer stainless steel sinks;
- three black polypropylene sinks, 100 cm long, 50 cm wide and 50 cm deep, fitted with large-bore constant overflow pipes and quick-release traps.

Recommended building specifications

It is understood that buildings and services should comply completely with national building standards; however, the following specifications are offered as a guide:

Walls. Load-bearing walls should be confined to external and corridor walls. External walls should be of either double brick cavity with damp course or hollow block concrete. The external walls should be finished with mortared brick or, in the case of hollow block concrete, finished with cement rendering.

Corridor walls and the walls of hot rooms and freezer rooms should be made of hollow cement block with both surfaces cement rendered to a smooth finish.

All wall surfaces should be sealed with polyvinyl acetate (PVA) sealer before painting. Washable plastic paint should be used for all areas. In the wet areas, such as the bottle cleaning and media preparation rooms, exposed walls should be tiled from the floor to 50 cm above bench height with ceramic wall tiles using waterproof tile adhesive and waterproof grouting compound. All tile-bench junctions should be sealed with mould-resistant silicone rubber mastic.

Ceilings. Ceilings should be fitted with a false ceiling of fire-resistant fibre or cement or plasterboard. All joints and cornices should be plastered over and sealed to prevent dust entry.

Floors. Floors should be constructed of damp-proof reinforced concrete covered with terrazzo screed or tiles with terrazzo screed carried 15 cm up the walls. In high-traffic wet areas, drainage slopes of at least 1:100 should be made leading to plastic or glazed earthenware open drains situated along outside walls and covered with removable cast iron grids. All drain exits to the main waste water system should be covered with vermin-proof traps.

Doors. Doors in the filtration rooms and bottle store should be a standard 90 cm wide. All doors opening on to the service corridor and those linking the sections should be 150 cm wide to allow for easy access and to facilitate the subsequent installation of large pieces of equipment such as autoclaves and spray-dryer.

Windows. Windows should be large enough to permit adequate natural lighting and should be placed at least 120 cm from the floor. Window frames should be constructed of hot dipped galvanized steel with glass panes completely sealed with putty. Individual window openings should be of sufficient size to serve as emergency exits in the event of a fire. All opening windows should be mosquito- and fly-proofed with netting screens.

Benches. All bench tops should be factory pre-formed and melamine-surfaced, with the long edge post formed. They should be constructed from high-density particle board 40 mm thick and preferably 90 cm wide. Workbench heights should be 85 cm except in special areas where they may have a reduced height of 70 cm to serve as writing tables or for the installation of bench-top-mounted laminar flow cabinets.

Cupboards, drawers and sink units. These should preferably be factory-prefabricated, user-assembled, flat-pack units constructed from 18-mm melamine-surfaced high-density particle board and

equipped with four plastic adjustable legs. These units can be used to support the bench tops giving ample kick space and allowing complete access for the cleaning of floor areas beneath and around them.

Sinks for the bottle cleaning unit should preferably be constructed from heavy-duty polypropylene, 60 cm long, 50 cm wide and 30 cm deep, with a top edge flange of 3 cm and large-bore drain fitted with a removable vertical overflow pipe to facilitate the continuous rinsing of glassware.

Shelves. Box-form shelves can be made from 18-mm melamine-surfaced high-density particle board, 30 cm wide, with supports at 50-cm centres. The walls should be drilled and plugged using plastic expandable plugs and the shelves attached using No. 8 woodscrews, through right-angled 3-mm mild steel fish-plates, firmly screwed to the back of the top inside corners of each 50-cm box-shelf partition.

Electricity supply. The unit should be surface rather than sub-surface wired using cables of appropriate cross-section to take the load with a safety margin of at least 30 percent. Surface-mounting permits easy access for electrical maintenance and also allows ready access for future modifications. All outlets, except in cases where three-phase 380/400 V, 50 Hz is required, should be 220/240 V, 50 Hz, 15 amp earthed sockets, mounted 15 cm above bench height and, in wet areas, outlets should be of the sealed, splash-proof type, fitted with residual circuit-breakers to provide full operator protection. Each section should be provided with a suitable subdistribution board equipped with miniature circuit-breakers adjusted to the correct safety amperage. The whole laboratory should be connected to an automatic start, standby generator. A large-capacity 10.5 kV voltage stabilizer should be installed to serve the whole unit and at least one outlet from this should be connected to each room to provide stabilized voltage for vital equipment where performance might be compromised by a fluctuating supply.

Dedicated lines (i.e. lines that are not shared by more than one piece of equipment) should also be installed to freezers, cold rooms and hot rooms to ensure their continuous operation.

Lighting. Laboratories should be adequately illuminated with fluorescent strip lights to give 400 lux at bench height.

Gas. Natural gas such as propane or butane should be reticulated via copper piping to the bottle cleaning and media preparation sections and should be supplied from a covered, well-ventilated central gas store, located outside the building.

Water supply. Cold water with a pressure of at least 2.5 bar should be reticulated via 1-cm galvanized steel pipes to all sections. The hot water required in the bottle cleaning section can be provided from electrically operated flash heaters or large-capacity 300-litre storage heaters.

Fire control. Fire extinguishers suitable for flammable chemicals, electrical and general fires should be located in each section.

First aid. A comprehensive first aid kit should be prominently situated in one designated room, with eyewash facilities in the media preparation room.

Modern cell culture technology for vaccine manufacture

P.J. Radlett

Since the early pioneering work on the mass cultivation of mammalian cells *in vitro*, many types of cells have been grown in a wide variety of culture systems. These have been used for the isolation and identification of viruses, the screening of antiviral and anti-cancer agents, the production of hormones, antibodies and other cellular products and, most important, the production of viruses and other organisms principally for the preparation of vaccines.

The wide range of uses for cell culture has resulted in the development of an equally wide range of cell cultivation systems, each being more or less appropriate for the required purpose. The number of cell cultivation techniques to have found broad acceptance for vaccine production is, however, much more limited, and this chapter will concentrate on these systems.

In general terms, the methods used for the production of culture media are similar, irrespective of the cell cultivation system used, but the methods appropriate to the selection and establishment of cell banks and for cell culture depend on whether the proposed cell substrate is a continuous line or derived directly from primary tissue and whether it will grow in free suspension culture or requires a suitable surface substrate for cell attachment.

An outline of the facilities required for the operation of cell culture units is given in the chapter, Basic laboratory services and media preparation for vaccine production, p.223. The scale of the vaccine manufacturing operation and the nature of the organism with which the vaccine is to be prepared will, however, have an impact on these requirements, in terms of the size and type of cell growth facility and the level of biological containment required for the associated antigen production area, of which the tissue culture unit may form a part.

SELECTION AND STORAGE OF CELL BANKS

Any cell substrate selected for vaccine production should be shown to be free from adventitious contamination. This includes bacterial, fungal and mycoplasmal contamination which would otherwise be likely to have deleterious effects on subsequent cell cultivation. Screening should also include the frequently less obvious contamination from agents, particularly viruses, which may persist in latent forms throughout the manufacturing process and cause disease or other adverse effects when injected with the vaccine into animals.

The selected substrate must also support the replication of the required organism to levels which are adequate and economic for vaccine production and should present the antigenic component in an immunogenic form and in a manner that permits its simple and cost-effective recovery for subsequent downstream manufacturing operations. Finally, the substrate and/or the components which may pass into the finished product must in themselves be intrinsically safe for the animals that will ultimately be the recipients, for the

operators and for consumers of meat and meat products from the tissues of vaccinated animals.

Over the years there has been much debate on the intrinsic safety of established cell lines. In the early days of tissue culture the predominant view was that primary cells were likely to be safer because they lacked the "immortality" characteristic of continuous cell lines and, hence, the apparent risk of associated malignancy. Experience has shown that, providing the characteristics of cell lines obtained from normal tissues are carefully monitored and controlled and that the manufacturing process effectively eliminates whole cells from the product, the risks associated with their use are negligible. Furthermore, it is much more difficult to manage a cell production system in which each batch of material represents a new threat of introducing deleterious agents than it is with a cell line where a master stock can be established and thoroughly screened prior to its use for vaccine production. Petricciani (1987) has reviewed the arguments relating to the use of primary and continuous cell lines and concludes: "It is difficult to argue, however, that one is not better off with a very well tested cell system" (i.e. as offered by a continuous cell system) "and a manufacturing process which can cope with even theoretically worrisome contaminants".

In many situations the testing to which the cell substrate must be subjected will be laid down by the control authorities responsible for licensing the product. In Europe, the United States and some other countries, there are established guidelines for these requirements (EC, n.d.a and n.d.b) and, even in situations where there is not a mandatory requirement, the guidelines provide a useful and sensible basis by which the manufacturer can ensure that the products manufactured are likely to be safe to use. Against such a background, the manufacturer will identify a cell substrate which enables the production of the required antigen. In many cases this will be based on what has already been established by developments elsewhere and the manufacturer will need either to establish a new master cell bank (in the case of continuous cell lines) or to identify a suitable source of primary tissue.

Clearly, the amount of effort required to complete all the testing necessary to comply with the requirements described results in established and tested source materials with a significant commercial value. Manufacturers are unlikely either to make such materials freely available or to disclose the details of their findings. There are however, international depositories of characerized cell substrates which are unlikely to include the formally registered and tested materials but from which samples may be obtained for a nominal cost. Such organizations publish detailed information on those materials that they are able to make freely available (PHLS, n.d.) and which may often provide a source for initial culture material.

Primary cell culture

Primary tissue from an acceptable source will need to be transported to the laboratory rapidly under cool or cold conditions. Some primary tissues may be stored frozen pending culture, but in other cases there is a significant loss of susceptibility and/or viability associated with this practice.

There have been many reported techniques for handling primary tissue, but essentially all of these techniques comprise excision of the appropriate material for culture, dissociation as far as possible into a monodispersed suspension using a proteolytic enzyme (usually trypsin) either by itself or in combination with a chelating agent such as ethylenediamine tetra-acetic acid (EDTA) and dilution into a suitable

growth medium. The techniques used for the dissociation and culture of a range of primary tissues are described in some detail in a number of publications, specifically in the manual edited by Kruse and Patterson (1973).

The cellular material so prepared may then require or tolerate some degree of amplification through a limited number of serial passages or transfers (usually only one or two) before evidence of senescence becomes apparent. Because of the limited life of such cultures it is not normally possible to lay down banks of substrate material and the antigen production process is usually confined to the use of simple culture flasks on a rather small scale. The amount of antigen required for a live virus vaccine is frequently much lower than that needed for an inactivated product. In consequence, this method of production may appear attractive for the preparation of live vaccines, but these are the products where the risks of live extraneous agents persisting into the final production stages are greatest. Because of the inherent risks arising from these cultures, vaccines produced in this way should be very carefully controlled prior to release. Nevertheless, where suitable continuous cell lines are not available the method still provides a satisfactory method of producing vaccines.

Continuous cell lines

When a suitable cell line for vaccine production purposes has been identified, it will be necessary to store an adequate number of ampoules of cells (as many as practicable) to establish a master bank. This bank can then be subjected to whatever testing is considered appropriate to demonstrate the suitability of the material for vaccine production purposes. From this master bank, subsidiary master and working banks can be established. An ampoule of cells can be revived from the working bank to initiate a cycle or series of cycles of production, followed by the revival of a further ampoule for additional production cycles. In this way the working banks can be made to last for a very long time before they become exhausted, when they can be replenished from the subsidiary master bank which, in turn, can be replenished from the original master bank. Such procedures make it possible to supply suitable cells on an almost indefinite basis, without the need for passaging them more than a fixed and limited number of times from the original tested material. Unlimited passaging of cells may (at least in theory) increase the risk of propagating cells with modified characteristics or of introducing adventitious contamination. A maximum of 20 passages from the master bank to the final production culture is normally considered acceptable and, in most well-managed production systems, this permits the production of several harvests without recourse to further stored material and enables production to continue for many years from the same basic cell stock.

The method of culture depends on the particular cell type and the scale and nature of the final production process. In most cases, however, either simple flat-bottomed flasks (for anchorage-dependent cell substrates) or simple stirred flasks (for cells that will grow in free suspension) are appropriate for both the production of cell banks and the preparation of seed material prior to transfer to the full-scale production system.

Cell storage

The cells held in the banks described above are almost invariably stored at low temperatures, either over or in liquid nitrogen, or at least below -65°C in a mechanical refrigerator. Doyle, Morris and Armitage (1988) have provided an excellent review of the process of

cryopreservation which encompasses both the theoretical and practical aspects of the problem.

The following points are also worth noting:

- Storage over liquid nitrogen is the most satisfactory method. Storage in mechanical refigerators may, over several years, lead to some deterioration in viability. Immersion in liquid nitrogen can lead to problems with the integrity of the seal and there are risks from alternation between liquid and vapour phases if the liquid level is allowed to fluctuate.
- Some form of cryoprotectant is required. Dimethyl sulphoxide is extremely effective but is toxic and the contact time between the agent and viable cells in the liquid phase must be minimized. Glycerol is also very effective as a cryoprotectant and, because of its generally lower toxicity, may be easier to use.
- Much has been written about the freezing rate of cells and how it should be controlled. Controlled rate freezing equipment is, however, very expensive and by no means all cell substrates require this level of sophistication. Frequently, ampoules may be successfully stored directly over liquid nitrogen. Alternatively, some degree of control may be obtained by cooling ampoules in stages, through 4°C, to -20°C, -65°C and finally to the vapour phase of liquid nitrogen. In most cases, the cells in ampoules should be thawed as quickly as possible and diluted out of the preservative maintenance medium.
- Whatever the storage system adopted, the contents of the freezer will ultimately represent a considerable investment in time, resources and production potential. The importance of reliable, well-maintained facilities cannot be overemphasized and, wherever possible, dispersion of the cell stock to more than one location, or at least to more than one freezer, is recommended.
- The volume and density of the stored cells are critical factors that should be carefully optimized. Small volumes will freeze and thaw more quickly but high cell densities may rapidly modify the cultural environment, particularly the pH, during preparation and freezing. Conversely, sufficient cells are required in each ampoule to seed the revived culture adequately and at a sufficient volume to permit rapid cell amplification to full production scale.

PRODUCTION OF CULTURE MEDIUM

Much work has been directed towards the definition of culture medium for the growth of mammalian cells. The objectives of this work have varied among research workers, some concentrating on the precise definition of the nutritional requirements of cells without the inclusion of ill-defined supplements, while others have been concerned to minimize the waste of expensive raw materials. In the former case there is the obvious potential benefit of eliminating the risks of introducing adventitious agents and even of untoward vaccinal reactions when the traditional supplements such as serum and peptones are used. Defined media formulations have not, however, generally found favour for commercial vaccine manufacture, because industrial-scale yields were often lower (sometimes much lower) than could be obtained when supplements were used. Alternatively, the formulations described contained various complex additives which were too expensive for routine large-scale manufacturing operations.

Similarly, attempts to minimize the cost of culture media have not resulted in satisfactory formulations, partly because

such formulations do not always provide comparable yields on serial passage over the full life of cells at the full scale of the manufacturing operation and partly because medium production costs are generally a very small proportion of the total manufacturing cost.

Water

Water is a most important constituent of the tissue culture medium and there is a requirement for good-quality water that is free from pyrogens and as free as practicable from contamination with bacteria, other organisms and impurities that may interfere with the cultivation of cells. Although impurities can be difficult or impossible to define precisely, suitable water may normally be prepared by ion exchange followed by either reverse osmosis or distillation. Assuming that a suitable primary source is available, the major problems are likely to concern the sterilization and maintenance of the purification equipment and the storage of the purified water.

The columns used for ion exchange and reverse osmosis cannot yet be simply and routinely sterilized by steam *in situ* and, in consequence, care has to be taken with the design and installation of the equipment itself and the operating and sterilization procedures. Furthermore, great care needs to be taken with the subsequent storage of water to ensure that recontamination, particularly with organisms that may produce pyrogens, does not occur. These aspects are considered in the chapter, General design and operating requirements for vaccine manufacturing establishments, p. 171.

Basic medium

The basic medium for cell culture almost always comprises a solution of sodium chloride supplemented with a range of inorganic salts to provide a balanced, isotonic medium with glucose as a carbon source. This solution is supplemented with a range of amino-acids and vitamins aimed essentially at providing all the nutritional requirements of the cells. To achieve satisfactory growth and the necessary cell densities for subsequent virus production, however, it is usually necessary to supplement the medium with an ill-defined peptone or animal serum or, most frequently, both. Although the precise formulations used for different products in different laboratories may vary extensively, most are variations of the early formulations published by pioneers in this field including Eagle (1955 and 1959).

The formulations used for cell maintainance during the virus culture phase are usually similar to those used for cell growth but for a number of systems it has proved possible to eliminate the serum from the formulation. It should not, however, be assumed that the virus replication phase takes place under serum-free conditions, since the carry over of residual medium from the cell growth phase can often be considerable.

Serum

As described above, the success of most veterinary vaccine manufacturing operations depends on the inclusion of animal serum, at least in the cell growth medium. Partly because of cost and general availability, serum of bovine origin is used almost exclusively. Many reports in the literature describe the use of newborn calf or even foetal bovine serum for cell culture. This is extremely expensive and wherever possible the use of the much less expensive and more readily available adult bovine serum is to be preferred. Whatever the type of serum used, this component is of major concern in respect to the accidental introduction of adventitious contamination. Attention has to be paid to ensure that the product is obtained only from

healthy animals, is collected and processed under clean conditions and is adequately tested to give an assurance of freedom from extraneous agents. Because of the disease risks associated with this material, in many situations it is now only acceptable to use serum from countries such as New Zealand or parts of Australia in which the disease status is deemed satisfactory. Since some residual serum will very often find its way into the final vaccine, care also needs to be taken over the compatibility of the serum source and the target species for which the vaccine is intended. There have been cases of hypersensitivity associated with the use of serum from one species for the production of vaccines to be used in another.

Whatever the serum source, incorporation levels of 8 to 10 percent are frequently used for cell culture and, where care has been taken to use only selected high-quality serum, this can sometimes be reduced to 4 percent or lower without reducing cell yields. In such cases the economic benefits of using a high-quality but more expensive serum source can be justified economically. Even so, serum may well prove to be the most significant cost component of the medium, frequently exceeding all the other medium production costs combined.

Peptones

The list of peptones or similar compounds which have been used in culture medium is long and for perhaps understandable commercial reasons manufacturers are frequently reluctant to divulge the precise composition, origin and method of preparation relating to their products. In consequence, there may be significant variation between two media reported to contain a given concentration of peptone. The concentrations used in cell growth and maintenance media are frequently in the range of 1 to 5 grams per litre of medium and this alone can represent a significant proportion of the total medium production cost. The risks associated with introducing adventitious agents to this type of product are probably much lower than those associated with serum because a combination of heat treatment and enzymatic digestion is usually required in the preparation of the material. This cannot be assumed, however, and steps should be taken to gain an assurance that the risks via this route are indeed minimal.

Medium sterilization

The composition of tissue culture media generally precludes sterilization by heat and, although there were some early attempts to sterilize media by chemical means, filtration is the method used almost exclusively today. Early filtration processes relied on asbestos containing depth filters, which have been adequately demonstrated to produce a reliable sterile product – provided that the level of contamination in the unfiltered medium is controlled within reasonable limits (Telling, Stone and Maskell, 1966). Such filters have proved very effective at handling the rather difficult serum containing media. Where the nature of the medium has resulted in very low filtration volumes in relation to the filter area used or where double filtration has been used to increase confidence in the sterility of the product some reduction in cell growth potential has sometimes been experienced.

A more recent development in the manufacture of such filter media has been the introduction of asbestos-free sheets, brought about by the perceived health risks associated with the manufacture of materials containing asbestos. Although such sheets are claimed to be of a sterilizing grade, this has not always been the case and caution should be exercised in using such materials for final sterilization.

More recently, cartridge-type filters have gained wide acceptance for the sterilization

of tissue culture media. As with all filtration processes there is a need to control contamination in the unfiltered medium to within reasonable levels – both to reduce the number of organisms presented to the filter and to minimize the build-up of toxins and the possible degradation of the medium prior to filtration. In order to obtain economic throughputs from sterilizing cartridge filters, some form of prefiltration is generally required. This may be provided by the use of depth filters or cartridge units with a lower rating than the sterilizing filter. The presence of serum in the medium makes the use of cartridge units more difficult and the level of prefiltration required in such situations can be quite critical.

EQUIPMENT

The equipment required for cell and virus cultivation depends on the particular processes employed and is likely to be of stainless steel, glass or disposable plastic construction. Experience has shown that, where appropriate for the process, the type of equipment employed for bacterial fermentations is, with minor modifications, also applicable to mammalian cell culture and virus production. The main features of suitable static process plant are good smooth finishes and freedom from crevices and imperfections. These are particularly important in relation to stainless steel welds. Adequate valving is also essential to ensure effective sterilization and to permit the proper drainage of liquors and venting of air. Well-drained, properly arranged pipe runs are also of great importance.

The design of equipment that is to be sterilized by autoclaving should follow the same basic principles in relation to crevices, drainage and air venting, and attention must be paid to the handling of such equipment under sterile conditions including, where appropriate, its connection to static plant.

Culture equipment will require facilities for environmental control or will need to be held in a controlled environment. In some cases this can be as simple as maintaining the culture in a temperature-controlled room or cabinet, while in other cases some direct control of the culture vessel will be appropriate. It was demonstrated many years ago that temperature control could be achieved effectively in static tanks by the provision of a temperature-control loop with a heating facility directly applied to the tank (Telling and Elsworth, 1965). Despite the simplicity of this method many workers fear the effects of local overheating and still prefer an indirect system of circulating warm water – even with its attendant complexity.

Fixed culture vessels are amenable to the convenient installation of additional environmental control facilties, but these add a degree of complexity that needs to be carefully balanced against the perceived benefits. Automatic control of pH, although certainly desirable, may not be essential for many processes and, in view of the relatively slow growth rate of the cell culture, manual adjustment at intervals may be sufficient. Some form of oxygenation may be required to maximize growth yields, but it may not be necessary to resort to automatic control in order to satisfy the culture's requirements. Modern oxygen electrodes may be more reliable than their predecessors but, nevertheless, add a degree of complexity which manufacturers may prefer to avoid. The relatively low metabolic rate associated with cell culture has enabled some operators to arrange a programme of manually adjusted sparged air rates sufficient to satisfy the culture's requirements.

The more sophisticated modern manufacturing procedures may require

significant downstream processing. Equipment for some of these operations, such as that for concentration by ultrafiltration, may not be amenable to sterilization by steam. In such cases a regime of chemical sterilization must be developed but this will always carry potential contamination risks. Care needs to be taken to minimize the proportion of the plant that requires chemical sterilization, and also over the interface between chemically sterilized and heat-sterilized parts of the plant.

CELL CULTURE AND ANTIGEN PRODUCTION

The basic methods employed for cell culture and antigen production depend on whether the cell source is dependent on a fixed substrate for growth and replication (anchorage dependence) or will grow in free suspension. Although the benefits of simplicity and easy scale-up associated with the operation of free suspension systems are considerable, the range of susceptible cell lines suitable for vaccine production is rather limited and many vaccine manufacturing processes are based on anchorage-dependent systems. In contrast, the use of suspension systems has permitted the bulk production of some vaccines, such as those against foot-and-mouth-disease (FMD) and rabies, which are required in particularly large quantities.

Antigens produced by culture may be used for the production of either live attenuated or inactivated vaccines. The culture methods used are likely to be similar in both cases but, because the effective dose of a live attenuated vaccine (which is subsequently amplified by replication within the tissues of the host) is generally much lower than for an inactivated vaccine (the antigen of which cannot be amplified within the recipient), the volumes required at the production stage are proportionately lower for live vaccines.

In many cases the virus is released freely into the maintenance medium following incubation of the virus culture. Where this does not take place it may be necessary to disrupt the cells by mechanical, ultrasonic or chemical means to release intracellular virus prior to proceeding with vaccine production. There are, however, situations where the whole cell containing the infectious agent is used for the production of live attenuated vaccines. In all cases except the last it will be necessary to remove the cell debris resulting from the virus replication phase prior to proceeding with vaccine manufacture, and this has been variously accomplished by sedimentation, centrifugation or filtration.

Anchorage-dependent cell systems

Conventionally, anchorage-dependent cells are grown on the flat sides of simple glass or plastic bottles. In such cases, the scale-up for bulk manufacture may require no more than the use of multiples of the largest practicable bottles that can be incubated in a conventional hot room. Many attempts have been made to increase the efficiency of such a process by increasing the surface area within each unit available for cell growth. The use of roller cultures enables almost all of the contained internal surface area to be utilized – as opposed to less than half in a conventional static culture – but introduces the additional complexity of the need for roller equipment. Nevertheless, facilities for handling large numbers of roller cultures have been developed and, for those cells that cannot be grown in free suspension, the technique probably represents the most popular approach to vaccine manufacture even today.

Additional surface area can be provided by the introduction of spirally wound plastic film within a rolling bottle or by configuring the static vessel as a series of flat plates. Such systems have been used

with varying degrees of success but introduce additional problems of complexity and, as efficiency (and hence cell density) itself increases the need for some degree of environmental control within the unit, further difficulties are raised. Although the literature contains many descriptions and reports arising from the use of such systems, or their many variations, in practice none of the systems described has rivalled the simpler static flasks or roller bottles used for bulk manufacture.

Another very attractive option for the growth of anchorage-dependent cells is the so-called microcarrier culture in which the cells are grown on the surface of beads which, in turn, can be held in free suspension (van Wezel, 1967). By this method, in theory at least, the benefits of simplicity, straightforward environmental control systems and the relatively direct approach to scale-up associated with deep suspension culture, become available for the growth of anchorage-dependent cells.

In practice, however, there are a number of problems yet to be overcome, which, despite the efforts of a large number of people, have so far prevented exploitation of the full potential of the technique. The difficulties appear to be associated with the transfer of cells and beads from one scale of culture to seed the next and with the establishment of cultural conditions that provide adequate mixing to maintain the beads in suspension and permit metabolic exchange – at the same time avoiding shearing the cells from the surface of the beads.

Much work has been carried out on these systems, and many techniques have been described for overcoming the limitations but relatively few have succeeded in establishing routine large-scale production systems using this method. Despite these difficulties, however, there have been some reports of success in large industrial culture systems, although understandably few details can be found in the public domain. Of the options available, the use of specially prepared sephadex beads as the carrier substrate has been the most successful and the technique may well be worth consideration for production at the semi-industrial scale of operation, in the range of 5 to 50 litres, where the important environmental parameters required can be controlled.

A number of other systems for the growth and maintenance of mammalian cells have also been described, for example the hollow fibre and perfusion systems reviewed by Tharakan, Gallagher and Chau (1988) and Feder (1988). These techniques are applicable to the field of very high-density cell cultivation, such as that used for the production of monoclonal antibodies, where the maintenance of cells over long periods is essential and the cell growth phase is less critical. The problems of environmental control can be considerable, particularly in the former case (maintenance phase) and the equipment necessary is quite complex.

Hollow fibre systems have almost certainly not been used (and are unlikely to find favour) for vaccine manufacture. Equally, perfusion systems are unlikely to be considered appropriate for classical virus production operations in which the cell is grown and infected and the virus destroys the cell and is released. For chronically infected cell systems, where the cell is not killed but leaks virus persistently over a relatively long period of time, such techniques may well be applicable but, as yet, do not seem to have been used in routine vaccine manufacturing operations.

All of the above options are in principle amenable to use with anchorage-dependent cells from any source, including both primary cell culture and continuous cell lines. However, where more than one virus vaccine is being made from more

than one cell type, precautions should be taken to segregate the manufacturing operations and operators in space and/or time to minimize the risks of cross-contamination at both the cell and the virus production stages. This aspect has been dealt with more fully in the chapter, General design and operating requirements for vaccine producing establishments, p. 171.

Whatever the cell system selected, almost invariably it will also determine the virus production system. In most cases the virus production phase comprises a change of medium, with or without a cell washing stage, and infection with an appropriate quantity of virus seed within the original culture vessel or flask followed by a suitable incubation period.

Suspension culture

Because suspension culture permits the growth of large quantities of material within a single culture vessel, in which environmental control is a relatively simple matter and which can be readily scaled to any convenient volume, it remains the method of choice for any cell or virus system capable of growth and replication in this manner. The scale-up of conventional stirred tank bioreactors has been dealt with in many reviews including Rokem (1988). The conventional stirred tank continues to be used extensively for the production of FMD (Radlett, 1987; Radlett, Pay and Garland, 1985) and rabies vaccines (Pay *et al.*, 1985), both of which have been produced in culture vessels with a capacity in excess of 1 000 litres, and its application in the veterinary field is only limited by the range of suitable susceptible cell substrates.

Care must be taken to provide a level of agitation that maintains the cells in suspension and permits efficient metabolite and oxygen transfer but avoids the damaging effects of excessive shear. The relationship between aeration, agitation and shear is a complex one and the damaging effects of aeration under some conditions have been well documented (Handa, Emery and Spier, 1987). As with the use of anchorage-dependent systems, the virus production phase is usually continued by removing the spent cell growth medium, replacing it with a maintenance medium and infecting the culture with a suitable volume of virus seed. After an appropriate incubation period the culture is harvested and processed further (Radlett *et al.*, 1972).

Another system which has been increasingly used in fermentation processes, including in some cases the production of cells in free suspension, is the airlift reactor. Essentially this is a gas/liquid contacting device, in which injected air causes liquid to circulate through an enclosed concentric tube within the reactor (Seigel, Hallaile and Merchuk, 1988). As applied to tissue culture, the main use of this method appears to have been for the growth and maintenance of hybridomas, where a requirement for relatively high cell densities results in a need for particularly efficient oxygen transfer. In principle, the technique should be appropriate for vaccine production but to date does not appear to have been widely adopted, presumably because the better-known stirred tank systems perform satisfactorily for this purpose.

CONCLUSIONS

During the last 30 years much work has been undertaken on a whole range of culture systems which have found varying degrees of application within the tissue culture field. The number of techniques that have found favour for the routine production of veterinary viral vaccines is, however, much more limited. This chapter has highlighted the various approaches that have been used to date and indicated

the level of application that these techniques have found. The techniques that have found lasting favour have tended to be the simpler, less sophisticated ones and these have been amply demonstrated to be capable of routine and reliable operation, frequently at a large or very large-scale.

As with all biological processes great care needs to be taken to define the operating parameters as closely as possible and to ensure that only materials of an appropriate quality are used in the manufacturing process. In addition, all stages of production, including the manufacturing environment, must be closely monitored and controlled. When attention is paid to these issues, modern manufacturing processes can produce high-quality, effective products reliably, consistently and in the quantities required for field operations.

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Fermentation technology for the production of bacterial vaccines

F. Bover

Much has been written about fermentation technology. Elaborate equations have been developed to describe agitation kinetics, mass transfer, energy transfer, etc. but very little has been published regarding the use of fermenters in vaccine production. In this chapter the discussion is limited to the practical aspects of selecting and optimizing fermentation equipment and fermentation systems for vaccine production.

The production of a bacterial vaccine involves growing a strain of the causative organism in artificial culture under conditions that maximize the level of the protective antigen(s). Each individual aspect of the production system needs to be carefully selected but it is the manner in which the individual aspects are combined that influences the level of success attained. The way in which the bacterial strain, the culture medium, the fermentation equipment and the fermentation parameters are selected is of critical importance and each individual selection will be influenced by numerous factors.

THE ORGANISM TO BE PRODUCED

The strain of bacteria should be antigenically consistent with strains found in the target animal population. With some species of bacteria little antigenic variation has been found and so it would be appropriate to use a strain of proven production performance. With other species of bacteria there is considerable antigenic variation and careful selection of a strain or strains relevant to the target animal population is essential. For example, *Brucella abortus* Strain 19 has been successfully used in many countries as part of eradication programmes whereas with *Bacteroides nodosus* (ovine foot-rot) ten or more antigenic variants have been identified in a single country. A knowledge of the situation in the target animal population must form the basis of any strain selection.

Once the strain(s) has (have) been selected it is essential to establish a seed lot system which should then form the basis for all future development and production work for the vaccine. Seed lots should be preserved by lyophilization or stored in a suitable cryopreservative at -70°C or in liquid nitrogen.

GROWTH MEDIUM

Standard texts provide a guide to the type of media best suited for the cultivation of various species of bacteria but the medium nominated for any bacterium should be regarded as a guide only. Improved growth and the production of protective antigen may be achieved by supplementing the base medium with animal or vegetable extracts, amino-acids, peptides, nucleotides or inorganic salts. The medium formulation should be optimized for each strain of organism being produced. A large range of concentrated and dehydrated media are available from established companies such as Oxoid, Difco, BBL and Merck. The convenience of such media and their relative consistency need to be balanced against their cost – it may be

appropriate to use media prepared at the vaccine production laboratory or, alternatively, a range of meat- and vegetable-based digests and infusions could be considered. Meat or organs from animals likely to be affected with bovine spongiform encephalopathy or scrapie should not be used for media preparation under any circumstances. Neural tissue from any source should not be used.

The ultimate selection should be made after careful evaluation of all such factors as the reliability and consistency of supply, cost and whether extra processing equipment and laboratory space are required. A regular review of the basis for the selection should be made but substantial changes in the medium formulation may have an impact on the efficacy of the vaccine and thus its registration status. The quantity of medium or medium ingredients held in stock should be determined after consideration of such factors as purchase lead time, projected requirements, cost, storage requirements and stability.

A clean and reliable supply of water should be available either for rehydration of dried media or for use in the preparation of digests and infusions. Where the quality or consistency of water from the local supply cannot be guaranteed it may be necessary to use distilled or deionized water. When considering the use of local water, such factors as the natural mineral content and the level of treatment with antibacterials (e.g. chlorine compounds) should be taken into account.

Media can be sterilized by heating (e.g. autoclaving) or filtration. Wherever possible, autoclaving should be used to minimize the possibility of survival of viral contaminants. This is particularly important in the production of live attenuated vaccines and the temperature and duration of autoclaving should be established after careful validation.

FERMENTATION EQUIPMENT

The selection of the actual fermentation equipment to be used will involve the careful consideration of all relevant factors: the required output will influence the size of the fermenter; the respiration requirements of the organism(s) will impact on the configuration of the vessel, the stirring system and whether or not an efficient system of aeration is required; and the level of instrumentation and control systems required also needs to be carefully evaluated. It should be clearly understood that the mere presence of sophisticated instrumentation and control will not guarantee successful fermentations. Indeed, in many laboratories efficient manufacture of vaccines is achieved with relatively simple equipment, but there are some basic parameters that should be well controlled, including sterilization, maintenance of freedom from contamination and temperature control. Where the organism being produced is zoonotic, for example *Brucella* spp. or *Leptospira* spp., extra attention should be paid to containment of the organisms and also to sterilization of exhaust gas.

Fermenters range in size from barely 1 litre to hundreds of thousands of litres. The selection of the appropriate size to use in vaccine production depends on anticipated yields, required vaccine volume, etc.

Fermenters can be constructed of a range of materials such as glass, glass-lined steel or stainless steel. Generally stainless steel, with the internal surfaces polished to the highest possible standard, is preferred. The most common shape for fermenter vessels is cylindrical. Although it is the normal convention to have a diameter-to-height ratio of 1:3, other configurations have been used successfully in vaccine manufacture.

It is essential to have reliable agitation and there are a large number of means of achieving this. Paddles mounted on a

revolving shaft are the most common system for agitation. The difficulties in effecting reliable sealing where the stirrer shaft penetrates the top or bottom of the fermenter have led to the development of magnetically driven stirring shafts which avoid the need for direct penetration through the vessel wall. Other forms of agitation include vibrating discs, airlift and sparging systems.

TEMPERATURE AND pH CONTROL

Precise control of temperature is essential. The most common means of controlling the temperature is by circulating warm water through coils mounted inside the fermenter or through a jacket integrated into the fermenter wall. Ideally the system chosen should be capable of controlling the temperature to ± 0.5°C.

If the medium is sterilized by autoclaving in the fermenter an efficient cooling system is essential. It should be capable of cooling the volume of liquid from 121°C down to 37°C in 20 to 30 minutes to avoid exposing the medium to elevated temperatures for longer than necessary. In areas with a dry and temperate climate an evaporator cooling system will provide a cheap and efficient system for supplying the cooling needs of a fermenter laboratory. In areas with a hot and humid climate a more elaborate cooling system will be required.

Many of the fermentations associated with vaccine production are enhanced by adequate control of pH. Before pH can be controlled it must be measured and this can be accomplished quite simply by testing samples using strips of pH paper. However, more sophisticated systems, which use sterile electrodes to provide continuous measurement, are relatively common. Similarly, the actual adjustment of pH can be achieved by a simple manual system or by a complex automatic one. Only detailed knowledge of the particular fermentation system will allow a decision to be made as to how complex or precise the pH control system needs to be.

Control of the level of dissolved oxygen similarly enhances aerobic fermentations but the measurement of dissolved oxygen requires relatively sophisticated equipment. The level of dissolved oxygen can be adjusted by varying the airflow, the stirrer speed and the head space pressure.

INTEGRITY OF THE SYSTEM AND CONTROL OF CONTAMINATION

A fermenter must be capable of maintaining its integrity for the duration of a fermentation. Inoculation and sampling systems must be such that they do not compromise the fermentation by allowing the introduction of extraneous organisms. A sensible approach is to test the integrity of the fermenter and the sterilization, inoculation and sampling systems by incubating a suitable test medium (e.g. soybean case in digest medium) and simulating inoculation and sampling. The test medium should remain sterile while being incubated in such a test for at least seven, and preferably 14, days and the integrity of the air filtration should also be checked in a similar manner.

CONCLUSION

The ultimate test that any fermenter must pass is whether it provides a reliable and efficient means for the production of the required antigen. To achieve this, meticulous attention must be paid to each aspect of the chosen fermenter and fermenter system.

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The use of lyophilization in the manufacture of vaccines

J.C. Mariner

Lyophilization, or freeze-drying, is a method of preservation that greatly enhances the storage life and portability of many otherwise labile microorganisms and biological products. In regard to attenuated live vaccines, the ability to freeze-dry a viable organism is often a crucial determinant of sustainable, cost-effective application in the field. To date successful lyophilization has been confined to viruses and bacteria. No technique for freeze-drying viable eukaryotic cells has yet been developed, and this has greatly limited the impact of attenuated protozoal vaccine strains and protozoal infection and treatment schemes.

The manner in which biologicals are lyophilized influences the quality, especially the potency and stability, of the final product. Research on lyophilization has been expanding in recent years and the processes involved are now relatively well understood. This has led to an enhanced ability to control the process, greater predictability and the potential for more stabile commercial products.

Unfortunately, lyophilization remains a neglected area in the practical world of vaccine production. Biological scientists frequently view lyophilization as the realm of mechanical technicians who, in turn, confine their interests to the functioning of the machinery. Worse yet, the processes involved are best understood from the viewpoint of physical chemistry, a subject not close to the heart of most biological scientists or mechanics. As a result, lyophilization is handled as a turnkey system based on an antiquated protocol of obscure origin in which the operator follows the instructions and everybody hopes that the vaccine batch survives the ordeal.

This chapter will present some basic guidelines and concepts fundamental to successful freeze-drying. A conceptual approach will be used wherever possible. Since lyophilization of vaccines is an interdisciplinary topic involving biology, physics and chemistry, no more than a basic college introductory course level of prior training will be assumed for any field.

STORAGE METHODS AND THE ADVANTAGES OF FREEZE-DRYING

The degradation of stored biologicals occurs through chemical reactions and the fundamental task in preserving biologicals for long-term storage is to slow degradative reaction rates. Reaction rates are dependent on the effective concentration of the reactants, their mobility or activity, and temperature. Water plays an important role in degradative reactions by mobilizing reactants and facilitating structural changes in proteins (Hageman, 1988) and for effective storage, water must be removed or immobilized without disrupting the functional structure of the biological material.

Freezing is an effective method of immobilizing water and other reactants which preserves structure and function even in eukaryotic cells. However, the necessary equipment is not very portable and maintenance costs are high owing to

the continuous consumption of energy. Drying at ambient temperatures is an effective means of preserving some biological materials – the product is portable and storage costs are low – but for many sensitive biologicals, the physical stresses of drying result in loss of structure and function.

Freeze-drying, drying from the frozen state, combines the advantages of both these processes. The water is first immobilized by freezing and then removed by drying under vacuum, at low temperatures and without loss of the stable frozen structure. The product retains its viability during the drying process and can then be stored at temperatures above freezing. Lyophilized materials are sealed under vacuum or with a dry inert gas, such as nitrogen, to remove the degradative reactant oxygen from the system.

OVERVIEW OF THE PROCESS

A production-scale shelf lyophilizer looks like a complicated machine but consists of three basic elements: a refrigeration system, a vacuum system for evacuating the chamber and a heating system. The dual function of the refrigeration system is to freeze the product prior to drying and to chill the condenser during lyophilization while the purpose of the heating system is to supply energy to the product. The lyophilization chamber contains a number of shelves that can be raised mechanically for stoppering the product under vacuum at the end of the run. Inside the shelves is a fluid circulation system and this fluid is either chilled by the compressor system during the freezing step or heated during lyophilization to drive the drying process.

A lyophilization run can be divided into three steps: freezing, primary drying (sublimation of ice) and secondary drying (desorption of water). In a typical run (Figure 5), the shelves are pre-chilled to the minimum possible temperature, usually in the neighbourhood of -45°C. In Figure 5 note the super-cooling phenomena in the product temperature curve (Arrow A), the initiation of primary drying after product temperatures are well stabilized at -40°C (Arrow B) and the effective end of primary drying manifested by the increase in product temperature and the drop in condensor temperature (Arrow C). In this cycle the vacuum was regulated at 100 torr (13 332 pascals) during primary drying and the transition to secondary drying. After primary and secondary drying, the vaccine harvest, combined with a stabilizing excipient, is distributed in vials, fluted butyl rubber stoppers are placed halfway in the neck of the vials and the material is loaded on to the shelves.

The product is now in direct contact with the chilled shelves and rapidly freezes. Once the product temperature reaches about -40°C, the cooling power of the compressor system is shifted to the condenser which is then chilled to a temperature of -55° or -60°C. Once the temperature of the condenser is lower than that of the product, the vacuum system is engaged and the chamber is evacuated.

At this stage primary drying has begun. During this phase the crystalline ice in the product is removed by the process of sublimation in which the product is allowed to warm slightly to a temperature just below its collapse temperature (usually about -30°C), water vapour then exits from the product and travels to the condenser where it is trapped as ice. As the water sublimes from the product, it takes thermal energy with it, thereby cooling the product. The energy of the sublimed water is released to the condenser during condensation and this tends to warm the condenser – the shelf heating system and the refrigeration compressor must work to counter these effects. After several hours all the ice has been removed from the product and the process of sublimation

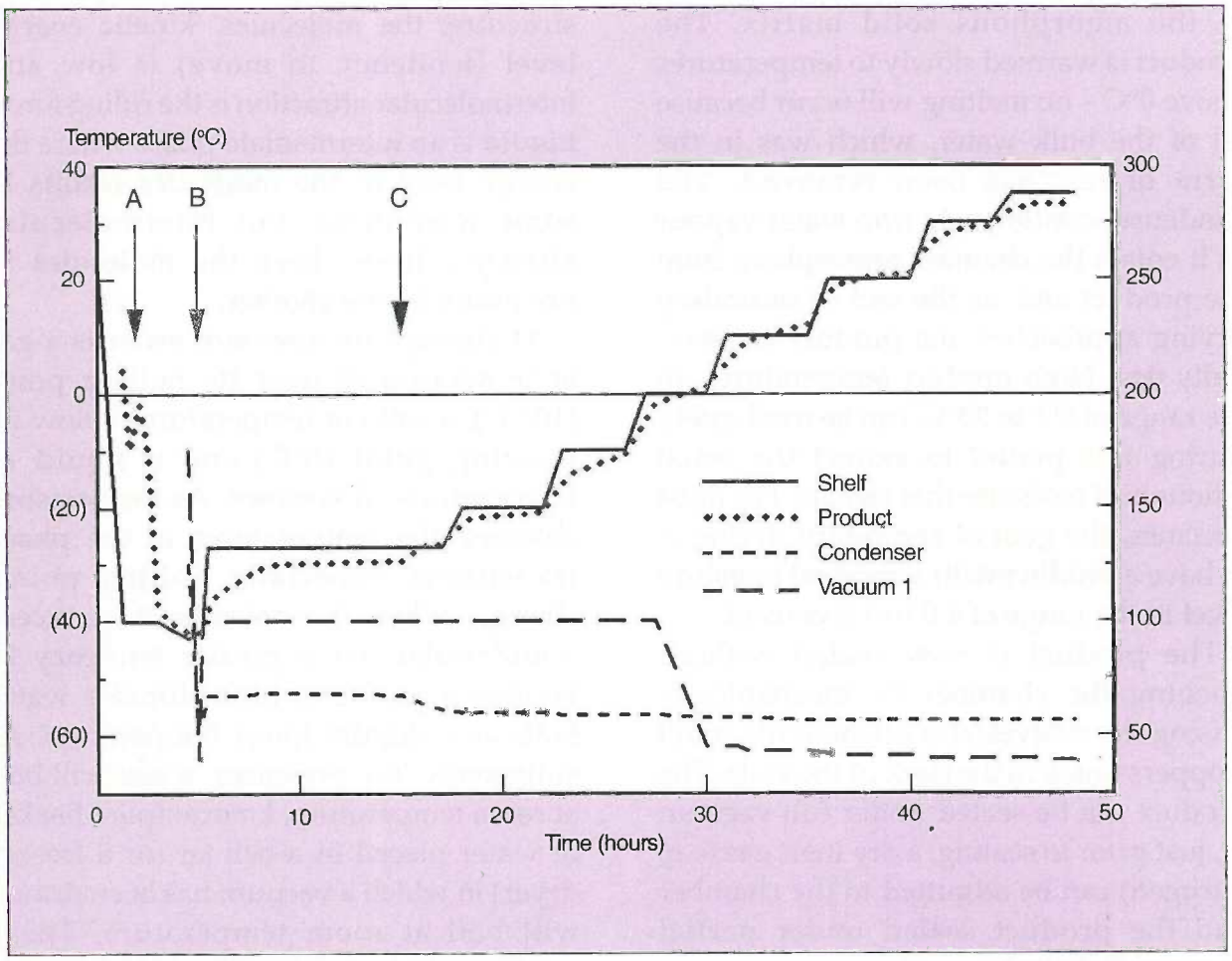

FIGURE 5
Sample lyophilization cycle

stops. The product now has a residual moisture content of 5 to 10 percent; the remaining water is not frozen but is absorbed into the solid matrix of vaccine and excipient.

Secondary drying can now begin. The function of secondary drying is to remove the majority of the absorbed water present in the amorphous solid matrix. The product is warmed slowly to temperatures above 0°C – no melting will occur because all of the bulk water, which was in the form of ice, has been removed. The condenser continues to trap water vapour as it enters the chamber atmosphere from the product and, as the end of secondary drying approaches, the product is essentially dry. High product temperatures, in the range of 25° to 35°C, can be used safely during this period to extract the small amounts of moisture that remain. For most vaccines, the goal of secondary drying is to have a product with a residual moisture level in the range of 1.0 to 1.5 percent.

The product is now sealed without opening the chamber by mechanically raising the shelves and pushing the butyl stoppers home in the neck of the vials. The product can be sealed under full vacuum or, just prior to sealing, a dry inert gas (e.g. nitrogen) can be admitted to the chamber and the product sealed under partial vacuum (0.8 atm).

BASIC PHYSICAL CONCEPTS OF LYOPHILIZATION

An understanding of the behaviour of water and solutions with water as the solvent is essential for successful freeze-drying.

Water exists in three phases: gas (vapour), liquid (water) and solid (ice). The phase of water is determined by the temperature and pressure conditions of the system. Temperature is a measure of the kinetic energy of the water molecules, i.e. their tendency to move. In the gas phase, molecules have complete freedom of movement and will distribute themselves evenly throughout a sealed container; their kinetic energy is high enough to overcome completely any intermolecular attractive forces. In the solid phase, the water molecules are tightly bound to one another in a highly ordered, crystalline structure; the molecules' kinetic energy level (tendency to move) is low and intermolecular attraction is the ruling force. Liquid is an intermediate phase where the energy level of the molecules results in some movement, but intermolecular attractive forces keep the molecules in proximity to one another.

At atmospheric pressure water is a gas at temperatures over its boiling point (100°C), a solid at temperatures below its freezing point (0°C) and a liquid at temperatures in between. As the pressure changes, the temperatures of the phase transitions, especially boiling point, change. When the pressure is reduced, liquid water has a greater tendency to become a gas, so at high altitudes water boils at a slightly lower temperature. At sufficiently low pressures, water will boil at room temperature, for example a beaker of water placed in a bell jar (or a freeze-dryer) in which a vacuum has been drawn will boil at room temperature. These relationships are best illustrated by the phase diagram Figure 6. Curves A-B and B-C are the vapour pressure curves for ice and water, respectively. Curve B-D is the vapour pressure curve for super-cooled water, a metastable state. From B-C, note that the boiling point drops with decreasing pressure. The phase diagram shows that water cannot exist as a stable liquid below a pressure of 4.58 torr (point B, the triple point). Below this point ice cannot melt, it can only sublime, as shown by the arrow.

Although most of the phase changes of water – freezing/melting, boiling/con-

densation and evaporation – are familiar from daily life, there is one phase change, sublimation, which may not be familiar since it occurs at very slow rates under atmospheric conditions. Sublimation is the transition of water molecules from the solid phase (ice) directly to a gas without passing through the liquid phase. Conceptually, sublimation is analogous to evaporation. Evaporation occurs from the liquid phase at temperatures below the boiling point. In the liquid phase, water molecules are continuously colliding and occasionally several molecules collide at one point and transfer all their kinetic energy to one molecule. When this happens near the surface, the molecule may break away from the body of the liquid and enter the gaseous phase. Sublimation can be viewed as the process of occasional molecules receiving enough kinetic energy to escape from the crystalline ice matrix and enter the gas phase. At low temperatures or low pressures, ice cannot melt, it can only sublime (Figure 6).

Sublimation is the critical process in freeze-drying. If the temperature is not allowed to rise and a vacuum is drawn (as in primary drying in a lyophilizer) the rate of sublimation will increase. As the water leaves the substrate, the frozen structure remains intact, the departing water molecules take their kinetic energy with them and, thus, the substrate is cooled. If the water vapour were not removed from the chamber, as the process continued water vapour would collect in the chamber atmosphere and eventually exert a pressure (the partial pressure of water vapour) which would result in condensation of water vapour on the product. This process would quickly reach an equilibrium with the amount of water subliming from the product being equal to the amount of water condensing on the product. It is the function of the chilled condenser to remove water vapour from the chamber atmosphere and, since the condenser is maintained at a lower temperature than the product, the water vapour preferentially condenses out of the chamber on to the condenser coils. Thus, an energy gradient is established from the heated shelf, to the product, to the chamber atmosphere and to the condenser.

FREEZING THE PRODUCT

The freezing of the product is a critical step. There are many methods of freezing but only one – freezing on the shelf of the lyophilizer – is practicable for large-scale vaccine production in developing countries. The rate of freezing is a major determinant in the structure of the vaccine cake owing to the fact that the freezing rate determines the size, shape and orientation of the ice crystals in the product and, after primary drying has removed the ice, the characteristics of the pores in the cake. During secondary drying these pores will act as the conduit for the desorbed water as it exits the cake.

Ice crystals form through a process of nucleation. In water solutions, the solute and impurities can act as nucleation foci and assist the process. As the product is rapidly chilled, the temperature of the solution falls significantly below zero before ice nucleation takes place; this phenomenon is called supercooling. At some point, nucleation takes place and, if the solution is sufficiently supercooled, the crystallization of ice rapidly spreads throughout the entire vial. As shown in Figure 5, the temperature of the supercooled solution returns to zero when nucleation occurs. As the crystals of pure water form, they exclude the solutes. The impurities, biological material and stabilizing excipients are concentrated in the interstices of the growing crystals and eventually these micro-environments become so concentrated that essentially no more water can be crystallized as ice.

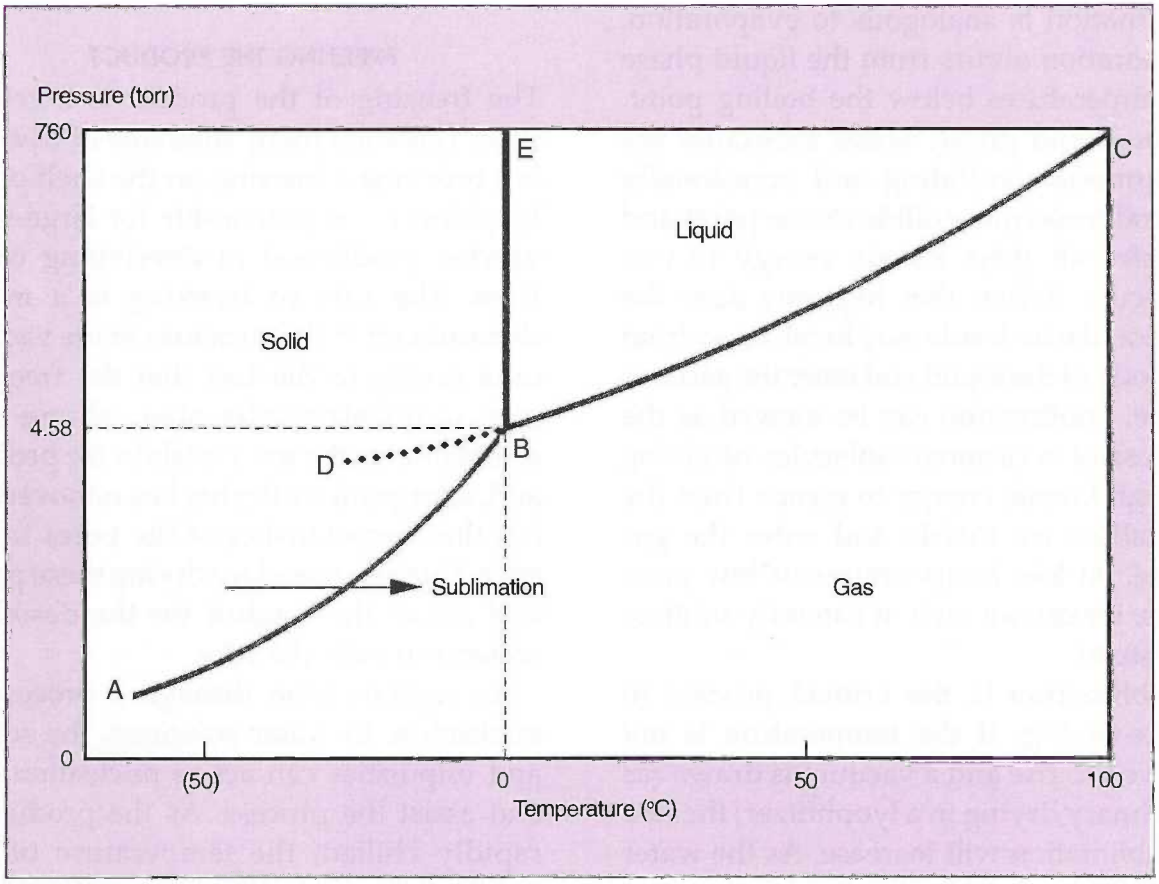

FIGURE 6
Phase diagram of water

During this process, two possible events may take place in the concentrated solute: the constituents of the excipient may form their own pure crystals; or the components may form an amorphous solid solution where the stabilizer components, biological material and unfrozen water are evenly distributed. The term amorphous means structureless and refers to a lack of regular crystalline structure. It is now commonly believed that crystallization of the excipient is undesirable, because if the stabilizer forms crystals it is not available for biological material and cannot perform the desired stabilizing interactions. A homogeneous amorphous solid is, therefore, the preferred structure.

Amorphous solids can exist in two states: rubber or glass. The glass state is an elastic solid whereas the rubber state is a deformable solid with a greater freedom of molecular motion and migration. The water present in the amorphous solid is not frozen and is said to act as a "plasticizer". The transition between the glass and rubber states is defined by an isoviscosity curve (Figure 7), the glass-to-rubber transition (T_g). As shown by the curve, the less water (plasticizer) present or the lower the temperature, the greater the tendency of the amorphous solid to be in the glassy state. Note that T_g' is the intersection of the solution freezing curve and the glass-to-rubber transition (T_g) curve. As the ice crystals form during freezing, the solute in the interstitial solution is progressively concentrated. The composition of interstitial solution can be thought of as moving from left to right across the bottom of the diagram until T_g' is reached. At this point no more water can be crystallized out of the interstitial solution and it becomes an amorphous solid. The water content of the amorphous solid is not frozen, as shown in Figure 7; it is in a metastable glassy state. The arrow indicates the portion of the T_g curve that is of interest during secondary drying and storage of the final product. As the curve shows, after the water content has been reduced to below 2 to 3 percent, the amorphous solid can support relatively high temperatures without collapsing.

The objective in freezing the product is to achieve a stable structure with the greatest immobilization of water possible and to maintain that structure through to the end of drying. Thus, the product must be chilled to a temperature below the glass-to-rubber transition temperature (T_g') of the solution.

In solutions containing one solute, T_g' is a characteristic of the solute and is independent of the initial concentration. In solutions containing multiple solutes, as with most bulk vaccines, T_g' is determined by the relative concentrations of the solutes and their respective T_g' values, i.e. the components of the immunogen harvest and stabilizing excipient. In practice, T_g' is above -30°C for most widely used veterinary vaccine formulations (Levine and Slade, 1988).

From this the following general guidelines for the freezing of vaccines can be formulated:

- Freezing should be as rapid as possible to ensure that the ice has a fine crystalline structure and the amorphous glassy material has a maximum specific surface area.
- The product must be frozen to a temperature below that of the glass-to-rubber transition of the amorphous solid component, i.e. to -40°C or the coldest temperature possible in order to insure that all vials in all locations are well below T_g'.
- In most vaccine production laboratories in developing countries, the highest freezing rate will be obtained by freezing the filled product directly on the shelf. The shelves should be chilled to the lowest possible tem-

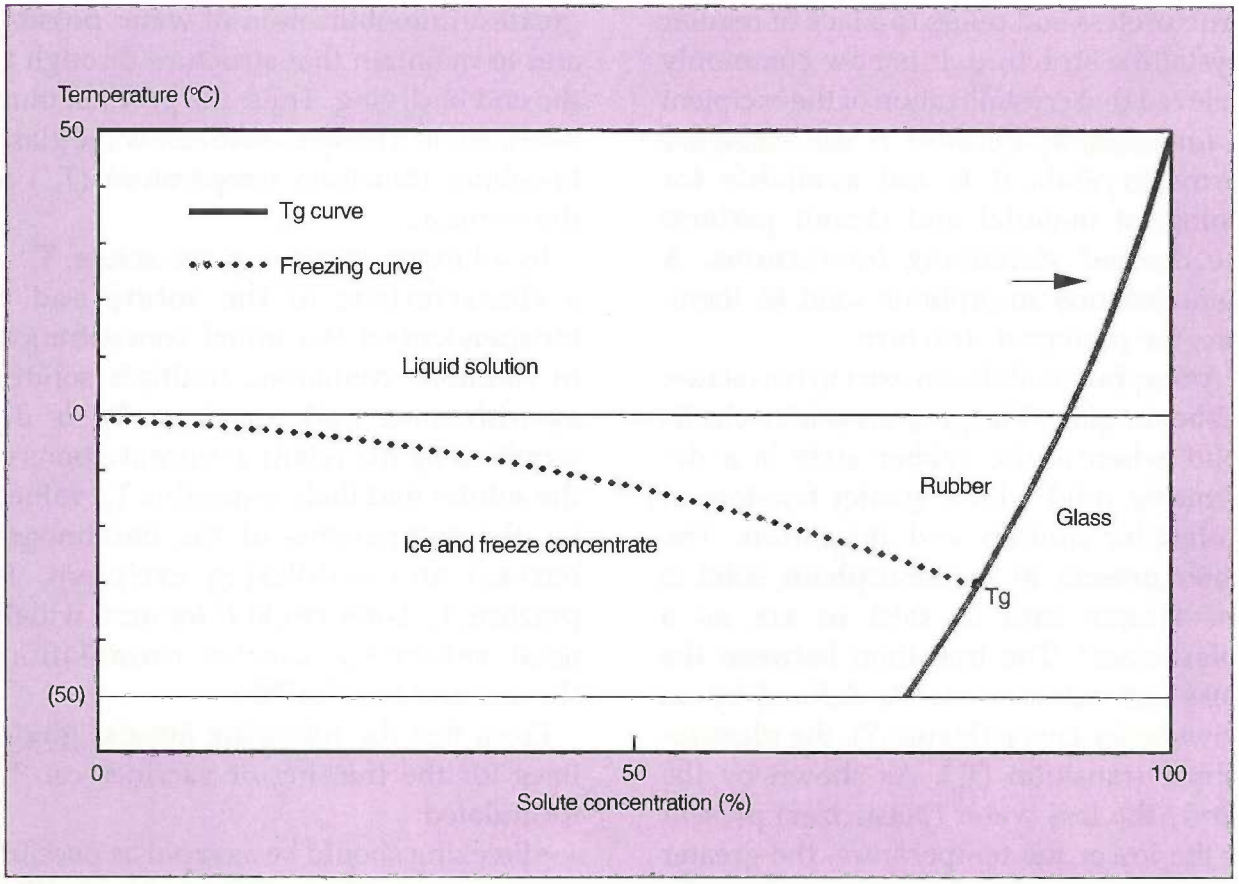

FIGURE 7
Glass/rubber transition state diagram

perature (usually -40° to -50°C) prior to loading. Pre-freezing should not be done in -20°C freezers or on inefficient pre-freezing racks. During the filling operation, filled trays can be kept cool in a +4°C walk-in refrigerator and then transferred to the chilled shelves of the lyophilizer.

PRIMARY DRYING: THE SUBLIMATION OF ICE

The product is now a mixture of ice and vitreous amorphous solid. The condenser is chilled to below the temperature of the product, a vacuum is drawn, the shelf heating system is engaged and ice begins to sublime. When the process is observed through the window a moving front of sublimation is visible descending from the top of the vaccine cake towards the bottom of the vial.

The critical concept for primary drying is collapse (melt-back). Collapse is the loss of the frozen structure, usually manifested by the destruction of the pore system left behind by the receding ice front. Once collapse occurs water is trapped within a shell of amorphous solid and effective freeze-drying is no longer possible. The resulting product will have a shrunken or foamy appearance and residual moisture levels will be higher than ideal for the stability of the final product. Collapse results when the product temperature exceeds the effective T_g' of the amorphous glass. When this happens the amorphous solid enters the rubber state and can flow while surface tension causes the fine filamentous structure of the amorphous solid to contract (Pikal and Shah, 1990). If the temperature rise above T_g' is extreme, the cake viscosity will be greatly reduced and foaming of the cake will be evident. This can be considered as being the boiling of liquid at low temperatures under a vacuum.

The fact that a frozen product can sit on a heated self and not collapse is one of the apparent paradoxes of freeze-drying and occurs for two reasons. The first reason is that sublimation during primary drying has a cooling effect caused by the fact that the water vapour exiting the substrate takes its kinetic energy with it. This is analogous to evaporative cooling and, if heat was not added through the shelf, the vaccine temperature would fall, the sublimation rate would quickly decrease to zero and drying would stop. The second reason is that the system is under vacuum and a vacuum is a good thermal insulator.

Heat can be transferred between two solid bodies by three methods: conduction; convection by an intervening gas or liquid phase; and radiation. Heat transfer between two bodies by conduction is the transfer of molecular kinetic energy (heat) through the collision of the molecules. This type of transfer can occur directly between two solids in contact but direct contact between solids is very limited – a minute intervening space is present between many of the surfaces that appear to the naked eye to be in contact. If this intervening space is filled by a gas, indirect conduction of heat can take place through the collision of the gas molecules with the two bodies and this greatly enhances heat transfer. Heat transfer by convection is the added effect of molecular flow or currents in the gas, thus, if the intervening space is a high vacuum, heat transfer by gaseous conduction and convection is severely restricted. If the lyophilization chamber is maintained under high vacuum during primary drying, appreciable heat transfer from the shelf to the product only occurs through the processes of radiation and conduction by direct contact and these are relatively inefficient methods of heat transfer.

In order for primary drying to proceed at an efficient rate, the product should be kept at the maximum safe temperature which is usually taken to be about 3°C

below the collapse temperature. To maintain this temperature while sublimative cooling is occurring, significant amounts of heat must be transferred to the product and at maximum vacuum this would require high shelf temperatures. High shelf temperatures pose a number of risks to the process, especially in developing countries where power cuts are common. If the vacuum system fails, sublimation stops, the product quickly warms to the shelf temperature, collapse occurs and the batch is lost.

It has been found, however, that if a moderate vacuum level is used sublimation continues unimpeded, but heat transfer by conduction and convection is increased. Many commercial-scale lyophilizers are capable of drawing a vacuum of 40 torr (5 333 pascals) or less which, at primary drying temperatures of -30°C, is far lower than ideal (normal atmosphere at sea level is 101 325 pascals). The vacuum level should be regulated to a pressure equal to one-half of the partial pressure of water vapour over ice at the product temperature used during primary drying (Le Floc'h, 1986). In practice, this is usually in the range of 100 to 120 torr (13 333 to 16 000 pascals) when primary drying is done at -30°C. Vacuum regulation is available as a standard feature on many commercial-scale lyophilizers and as an inexpensive option on almost all modern models.

The following are guidelines for safe and efficient primary drying:

- Primary drying should be done at a product temperature 3°C below the collapse temperature (essentially T_g').
- For most veterinary vaccine preparations, primary drying can be done at temperatures of -30°C or above.
- A vacuum should be maintained at 100 to 120 torr (13 332 to 16 000 pascals) during primary drying at product temperatures of -30°C or above.
- Where good temperature control is available, shelf temperatures in the range of 3° to 4°C above the desired product temperature should be used, especially when the vacuum is regulated. This reduces the risk of product collapse and eases the transition to secondary drying.
- Primary drying can be considered to be at an end when no visible ice is present in the product, the product temperature begins to rise to the level of the shelf temperature and the condenser temperature falls (Figure 5).

SECONDARY DRYING: THE DESORPTION OF WATER

At the end of primary drying, the product cake is a porous filamentous glass with between 5 and 10 percent residual moisture. All of the crystallized water (ice) has been removed. The remaining water is absorbed within the amorphous solid and the objective in secondary drying is to remove most, but not all, of the remaining water. A certain amount of water (generally 1 to 1.5 percent residual moisture) is essential for the structural integrity and viability of live vaccines but more than this minimal level is detrimental to product stability.

At the beginning of secondary drying, the collapse temperature of the amorphous solid is still essentially equal to T_g'. As water is removed from the glass, the glass transition temperature increases (Figure 7), thus, progressively higher temperatures can be used during secondary drying without causing product collapse.

Pikal *et al.* (1990) have shown that the important determinants of secondary drying rates are the specific surface area of the amorphous glass and the shelf temperature. Their study found that products rapidly reached a plateau moisture level at a given temperature and that further drying was minimal if the product was left

at that temperature for a prolonged time. Final shelf temperatures are thus a major determinant of product residual moisture content and the higher the final shelf temperature the drier the product.

In practice, many manufacturers go to high shelf temperatures (+25°C) relatively rapidly. However, exposing a humid product to high temperatures runs the risks of causing partial collapse of the product and of significant losses in potency. A more prudent procedure which also takes advantage of high final temperatures is to increase the shelf temperature step by step. At each successive temperature step, product moisture content reaches a lower level and product temperature can safely be increased to the next step. Application of this technique to rinderpest vaccine stabilized with 5 percent lactalbumin and 10 percent sucrose allows final shelf temperatures of 35° to 40°C to be used with minimal loss in product titre (> 0.3 $\log_{10}$ $TCID_{50}$). Although the stabilizer is highly hydroscopic, the residual moisture content of the final product is between 1 and 1.5 percent and product stability is sufficiently enhanced to allow use of the vaccine with a reduced cold chain (Mariner *et al.*, 1990).

It is also common practice to draw a full vacuum during secondary drying and some computer-controlled freeze-dryers have made this obligatory. Drawing a full vacuum, however, does not necessarily accelerate secondary drying (Pikal *et al.*, 1990).

The following are guidelines for safe and efficient secondary drying:

- Increase shelf temperatures in steps, allowing at least two hours at each step.
- The product temperature should rapidly equilibrate with or surpass the shelf temperature during secondary drying, otherwise significant product moisture may be present and product collapse (shrinkage) may result.
- Final shelf temperature is a major determinant of product residual moisture content and, thus, product stability.
- For most veterinary products, the final shelf temperature should be in the range of 25° to 35°C.

SELECTION AND PREPARATION OF PACKAGING

All of the effort put into freeze-drying is lost if the quality of the packaging is inadequate. Good-quality packaging assures more uniform heat transfer and drying as well as providing a robust seal capable of maintaining a vacuum over a range of temperatures. Vials and stoppers should be considered as two components of one system; the stopper must be matched to the vial. Vials should be thin and of uniform thickness to allow for rapid, uniform heat transfer. Vials manufactured from tube glass are ideal and can be obtained at prices comparable to moulded glass vials. Vials should not be recycled as complete cleaning is virtually impossible and microscopic contamination of the neck can result in microleaks during storage. New vials should be stored in a dust-free environment and rinsed with distilled water prior to use. For sterilization, the simplest and safest method is to load the vials on to the lyophilizer trays, wrap the trays in foil and sterilize in a dry oven. In this way, the sterile trays can be filled with product with a minimum of handling.

Stoppers should be coated with silicone to assist in the stoppering process at the end of lyophilization. The only function of the silicone is to reduce friction, and excess silicone may compromise the stability of the seal or contaminate the product. Properly siliconized stoppers have no visible silicone, they just feel slightly slippery. Many manufacturers provide pre-siliconized stoppers which are ideal. If silicone must be applied during pre-

paration, spread the stoppers on a tray and either spray them lightly with an aerosol preparation or place a small amount of silicone on the hands and work the stoppers. Using either method, the stoppers should be thoroughly mixed by hand to assure a thin, even coat.

The stoppers are then autoclaved at 121°C during which they absorb a considerable quantity of moisture. This moisture will not be removed during lyophilization but will equilibrate with the product cake during storage resulting in a significant rise in residual moisture and loss of stability (Held and Landi, 1977; Le Meste *et al.*, 1985). Thus, a well-dried final product with 1 percent residual moisture at the time of exit from the apparatus can have residual moisture of 5 percent or more after six months storage at 4°C if the stoppers are used directly from the autoclave without drying.

It is recommended that stoppers be subjected to four hours of drying at 135° to 142°C in a drying oven just prior to use. These drying conditions will not damage the stoppers but temperatures and times in excess of these may cause them to harden and lose their "rubber" sealing properties. Stoppers should be packed in cotton sacks or in another porous material in moderate quantities to ensure adequate drying throughout. Vacuum-drying in the autoclave cannot be recommended as a general procedure as the efficiency of this treatment varies greatly between machines (Mariner and Sassu, 1993). There is also limited data to suggest that stoppers made from chlorbutyl are superior to those made from isobutyl in terms of reduced moisture absorption and desorption.

The following are guidelines for the selection and preparation of packaging:

- The use of new, tube glass vials with matching stoppers is highly recommended.
- The integrity of the vacuum seal should be evaluated under conditions of temperature stress. A simple test is to submerge a sample of the final product in a water bath at 45°C for 24 hours and observe the cake for evidence of contamination with aspirated water.
- Stoppers should be dried at 135°C for four hours following autoclaving.

PRODUCT FORMULATION

The content of the product profoundly affects the physical processes of lyophilization. In veterinary vaccines, excipients tend to be composed of a protein, a sugar and a buffer system. An ideal excipient would have the following properties:

- immunological and pharmacological inertia;
- it stabilizes the biological during freezing, drying and storage;
- it dries readily at moderate primary drying temperatures without collapse;
- low cost and ready availability;
- it is easily sterilized;
- it rehydrates rapidly;
- it has an elegant appearance.

Unfortunately, the ideal stabilizer has yet to be found. As an example, skim milk powder used in the production of contagious bovine pleuropneumonia vaccines does well in all categories except that it is difficult to sterilize. Disaccharides such as sucrose, lactose and trehalose work well in viral vaccines but require longer drying times at optimum concentrations for stability and, in addition, trehalose tends to add to the cost of the product.

Freezing and drying have been shown to be different stresses and the mechanisms of stabilization are becoming clear. During the freezing of proteins, any solute that is preferentially excluded from the surface of the protein results in a protective shell of hydration and, owing to the non-specificity of the interaction, a wide range of additives exhibit cryostabilizing effects. During drying more direct interaction

between the stabilizing additive and proteins is required. Disaccharides perform well as stabilizers of proteins during freezing and drying (Crowe *et al.*, 1990) and have also been shown to stabilize phospholipid membranes during drying (Crowe *et al.*, 1987). Disaccharides are able to stabilize proteins during drying by replacing water at hydrogen binding sites. They are also believed to stabilize membranes by forming favourable interactions with the polar head groups of the phospholipids, thus conserving the proper intermolecular spacing between the phospholipids after the removal of water. Monosaccharides such as glucose are able to stabilize proteins during freezing by the preferential exclusion mechanism, but are unable to take on the hydrogen binding role required of a good drying stabilizer. For freeze-drying, stabilizers that protect against both freezing and drying stresses are necessary and disaccharides are the biological stabilizer of choice.

The role of the protein component is to increase the structural stability of the cake, protecting it against collapse. Live vaccine preparations would have low protein contents if additional protein were not added. On average disaccharides have low T_g' values (about -30°C) whereas proteins tend to have moderate T_g' values (about -10°C). Excipients that combine a protein with a stabilizing disaccharide result in a solution with a T_g' value in the range of -15° to -25°C, a more practical drying temperature in commercial-scale freeze-dryers. For extensive tables of T_g' values, see Franks (1990) or Levine and Slade (1988).

The following are guidelines for the selection and use of excipients:

- For live veterinary vaccines, excipients should contain a protein, a disaccharide and a buffer system.
- The collapse temperature of the cake is determined by the relative concentration of the excipient components. In less efficient lyophilizers the risk of collapse can be reduced through the selection of components with high T_g' values or through changes in the relative concentration of components.
- Whenever possible excipients should be added at the time of harvest, prior to freezing, to take advantage of their cryoprotective effect.

QUALITY CONTROL OF LYOPHILIZATION

The objective of freeze-drying is to increase the shelf-life of the product while maintaining the purity and potency of the bulk harvest. The quality control tests which relate directly to the efficiency of the lyophilization are the titration of the vial filling pool and the final product, the measurement of residual moisture content in the final product, vacuum testing and accelerated stability testing.

Titration procedures for the major veterinary vaccines are already well described (PANVAC, 1993). The important parameter for assessing freeze-drying efficiency is the loss during lyophilization or the difference in titre between the filling pool and the final product. In rinderpest vaccine production these losses are typically in the order of 1 $\log_{10}$ $TCID_{50}$ per millilitre, however a more appropriate freeze-drying cycle can reduce these losses to less than 0.3 $\log_{10}$ $TCID_{50}$ per millilitre (Mariner *et al.*, 1990).

A number of techniques are available for the measurement of residual moisture (May *et al.*, 1982). These techniques measure different forms of residual moisture and, in practice, no technique measures absolute water content. It is difficult to compare residual moisture measurements made using different testing procedures, but in-house standards can and should be developed based on one reproducible technique. In the past, a quantitative chemical method of measuring

residual moisture, the Karl Fischer test, was introduced into several laboratories in developing countries. Unfortunately, the Karl Fisher test is not very objective, is highly sensitive to moisture contamination and involves the use of absolute alcohol which is difficult to ship. Gravimetric and thermogravimetric tests are simple, accurate techniques applicable to laboratories in developing countries.

In the gravimetric test, vaccine is weighed, placed in a vacuum desiccator for a period of two weeks and then reweighed. The residual moisture is calculated as the weight loss divided by the initial weight of the vaccine expressed in percent. This technique has the advantage of requiring simple apparatus normally available in vaccine production laboratories: a sensitive balance (0.1 mg), a vacuum pump and a desiccator. The thermogravimetric method was originally developed for residual moisture measurement using a vacuum oven. However, a wide range of thermogravimetric balances are now available for measuring residual moisture. These devices combine a balance with a heating element and allow the residual moisture test to be completed within two hours. Their cost is comparable to a good balance and most can be used as a balance when not measuring residual moisture. Many of these devices are designed for measuring the moisture in foodstuffs, etc. where large sample sizes are affordable (10 g). Institutes purchasing a moisture measurement balance for vaccines should select one capable of working with 1 g samples (a 1-mg or 0.1-mg balance) and good temperature regulation in the range of 60° to 70°C.

The recommended temperature for measuring residual moisture in vaccines without charring the sample is 60°C. The moisture analyser can be set to this temperature and left until the sample weight (moisture content) becomes constant. This can take several hours and protocols for measuring moisture at higher temperatures (70°C) have been developed which greatly reduce the test time. High temperature protocols must be validated by testing samples in parallel at 60°C.

The recommended method for determining product stability is accelerated stability testing. In this test, the product is placed at elevated temperatures, usually 37° or 45°C, and samples are taken over time. The data is then plotted with titre on the Y-axis and time on the X-axis – if the data is linear or can be segregated into linear components the slope of the line can be determined using linear regression. This slope is termed the degradation constant (k) which is the best measure of vaccine stability and is independent of many non-stability parameters such as initial titre. By comparing k values for a specific temperature for vaccine batches produced under differing lyophilization protocols, the relative effect of the protocol on vaccine stability can be determined. This is an essential step in product development.

There are also more advanced applications of k values and accelerated stability testing. If measurements of k are made at three temperatures (e.g. 37°, 45° and 56°C) the three k constants can be related using the Arrhenius equation and the slope of the Arrhenius equation can be used to predict the value of k at lower temperatures. The advantage of this procedure is that vaccine stability can be predicted at moderate temperatures such as 4° and 10°C where tests would have to be prolonged over several years to observe an effect. Mariner *et al.* (1990) provide an example of the application of this technique to rinderpest vaccines.

The following are minimum guidelines for quality control of the lyophilization process:

- Determine the loss of potency during lyophilization.

- Where vials are sealed under vacuum, spark-test a random sample of the final product.
- Measure the residual moisture using a recognized method.
- Determine the degradation constant (k) at 37° or 45°C on representative lots of vaccine for each product formulation and/or freeze-drying protocol.

STORAGE OF LYOPHILIZED PRODUCTS

As a general recommendation, live lyophilized veterinary vaccines in stoppered vials should be stored at temperatures between +4° and -20°C. Lyophilized products in stoppered vials should never, under any circumstances, be stored at temperatures below -20°C. The glass-to-rubber transition temperature for butyl stoppers is about -70°C which means that at these very low temperatures the stopper will be in the glassy state and lose all of its desirable sealing properties. Furthermore, if the stoppers are not adequately dried, the water content may cause them to lose their elasticity at more moderate temperatures such as -30° and -40°C. Thus, at temperatures below -20°C, the stoppers will begin to harden and loss of vacuum is a very real risk.

Butyl stoppers are relatively impermeable to water and gas at low temperatures, but diffusion can take place over a period of years. Vaccines should be stored in a dry environment to reduce the risk of moisture contamination. Light can also cause significant degradation of the product, particularly in the case of rinderpest vaccines, and all vaccines should be stored in the dark.

CONCLUSION: A NOTE ON VACCINATION ECONOMICS

Freeze-drying is a critical step in the vaccine manufacturing process. Product formulation as well as the time, temperature and vacuum settings used during lyophilization affect product potency, stability and portability.

With the current emphasis on privatization and cost-recovery, minimizing production costs is an important concern. Eliminating unnecessary expense will be a prerequisite for survival in a competitive private market, but maintaining or improving the quality and presentation of the product will be equally important. Many producers in developing countries are obsessed with shortening lyophilization cycles and skimping on the cost of vials, but this is a false economy.

The estimated cost of vaccinating an animal against rinderpest in Africa ranges between US$0.30 to $0.75. However, it is important to remember that vaccine costs represent less than 10 percent of the cost of vaccinating an animal and that vaccine quality is the single most important determinant of vaccination success or failure. A poorly packaged and lyophilized product may be a cent cheaper per dose, but is much more prone to cold chain failures. Veterinarians and paying consumers will insist on products that work and are cost-effective in the field. The optimal efficacy and portability of a well lyophilized product is essential for success in the market.

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Inactivation of antigens for veterinary vaccines (1)

Viral vaccines

H.G. Bahnemann

For many years antigens for viral vaccines were inactivated by treatment with formalin (an aqueous formaldehyde solution) but, as early as the 1950s, suspicions were being voiced that formalin-inactivated foot-and-mouth disease (FMD) vaccines had, in some cases, caused the disease after application, although the same vaccines had passed official innocuity tests (Lucam, Mackowiak and Magot, 1958)

In studies of the inactivation of poliovirus with formalin in the years 1955 to 1958 it was shown that the inactivation process is complex and not a first-order or linear reaction (Gard and Lycke, 1957). Formalin reacts with many chemical groupings of proteins, which leads to the phenomenon of the "membrane effect" in which the reactions "close" the outer protein shell of the virus before the nucleic acid of the infectious genome has been destroyed. Even after prolonged incubation of the inactivated antigen, infectious nucleic acid can emerge and lead to a replication of the virulent virus. This can cause a subclinical infection or even lead to disease in the vaccinated animal.

The membrane effect alters the surface proteins of the virus and modifies and reduces the antigenicity of the antigen. It also stabilizes the antigen.

In the late 1950s and early 1960s a new inactivant for FMD virus was reported in the literature (Brown and Crick,1959) and used later by a leading FMD vaccine manufacturer. This inactivant was N-acetylethyleneimine, or AEI, which belongs to the group of alkylating substances.

It was shown that the inactivation curve produced with this substance was linear (Graves and Arlinghaus, 1967) which meant that the inactivation reaction was more specific for the nucleic acid. The inactivation rate and end point could be calculated, and this was a great improvement in the control of the inactivation process. It was also demonstrated that the inactivating agent produced an antigenically superior vaccine (Girard *et al.*, 1977) owing to the fact that alkylating substances react very little with proteins and therefore do not alter the antigenic components of the virus.

Nevertheless, AEI was patented, which prevented a more widespread application of this inactivant. Other alkylating substances were therefore investigated, such as ethyl-ethyleneimine (EEI) (Bauer, 1970) and ethyleneimine (EI) (Bahnemann, 1973). They also inactivate in a linear mode but were not readily available for large-scale application and had the disadvantage of requiring the handling of a highly toxic liquid.

A preparation of ethyleneimine – binary ethyleneimine (BEI) – was described slightly later (Bahnemann, 1975). It was produced by cyclization of bromo-ethylamine HBr (BEA) under alkaline conditions (NaOH solution). The cyclization process proceeds with a considerable pH drop from about 13.5 to 8.5 which can be followed and visualized by use of the pH indicator β-naphthol violet (BNV). BEA is used at a low concentration and this results in a concentration of 0.5 percent of EI and means that BEI is much less

dangerous to handle although reasonable caution must still be used in its preparation and application.

The three substances required for the preparation of BEI – BEA, NaOH and BNV – can readily be obtained from several chemical supply companies and are not expensive.

For all these reasons the application of BEI in the preparation of veterinary viral vaccines has become widespread. The largest application is in the preparation of FMD vaccines but many other viral vaccines for animals have also been produced with BEI as the inactivant (Bahnemann, 1990).

THE PREPARATION OF BEI AND ITS USE AS AN INACTIVANT

The preparation of BEI is fairly simple and straightforward. The first step is to prepare a 0.175 N solution of NaOH. It is strongly advised that this solution be prepared from NaOH pellets and with distilled water, always on the same day that the BEI preparation is to be made. In order to indicate the pH drop and control the cyclization of the BEA, a solution of 1 percent BNV in distilled water is prepared. From this stock solution 0.5 ml is added per litre of the 0.175 N NaOH solution. The resulting colour is a pale violet.

The BEA salt is then dissolved in the prewarmed (37°C) NaOH solution to a concentration of 0.1 molar (M) (20.5 g BEA per litre of 0.175 N NaOH). The cyclization to BEI is allowed to proceed for at least 30 minutes. After about 15 minutes the colour of the solution should have changed to a pale orange and if there is no colour change after about 30 minutes the solution should not be used for inactivation and should be discarded.

The 0.1 M BEI solution is then added to the virus suspension at a concentration 1 to 3 percent. This gives a final concentration of 1 to 3 millimolar BEI. Care must be taken that the virus suspension is already at the selected inactivation temperature (whether 26°C, 37°C or another) before the BEI is added under slow stirring.

The inactivation should proceed in two phases. The first phase, occupying perhaps a third or even half of the total inactivation time, occurs in the first vessel where the BEI was added and the inactivation began. For the second phase, the virus suspension should be transferred through a valve in the bottom of this vessel to a second vessel. This procedure is important to avoid any accidental recontamination of the inactivated virus suspension with infectious virus remaining on the vessel wall above the liquid level, or in dead spaces (dead-end tubes, valves etc.) of the inactivation vessel, after neutralization of the BEI.

Residual BEI has to be hydrolysed after inactivation. This can be done immediately after inactivation or after storage of the inactivated antigen and before vaccine formulation. The hydrolysation is carried out with a sterile 1 M Na-thiosulphate solution, which is added at equimolar concentration (to BEI), i.e. at 10 percent of the volume of the BEI solution used. The thiosulphate solution can be sterilized by autoclaving.

INACTIVATION CONTROLS

For each batch of virus supension, the inactivation rate (decrease of viral infectivity) should be calculated to allow the determination of the actual end point of the inactivation process. In the industrial production of viral vaccines, the inactivation rate can be expressed in log units of virus infectivity inactivated per hour.

The inactivation rate is calculated from infectivity titrations of samples taken after different intervals from the beginning of the inactivation process, for example, after one, two, three and four hours. From the values of the infectivity titres, the slope of

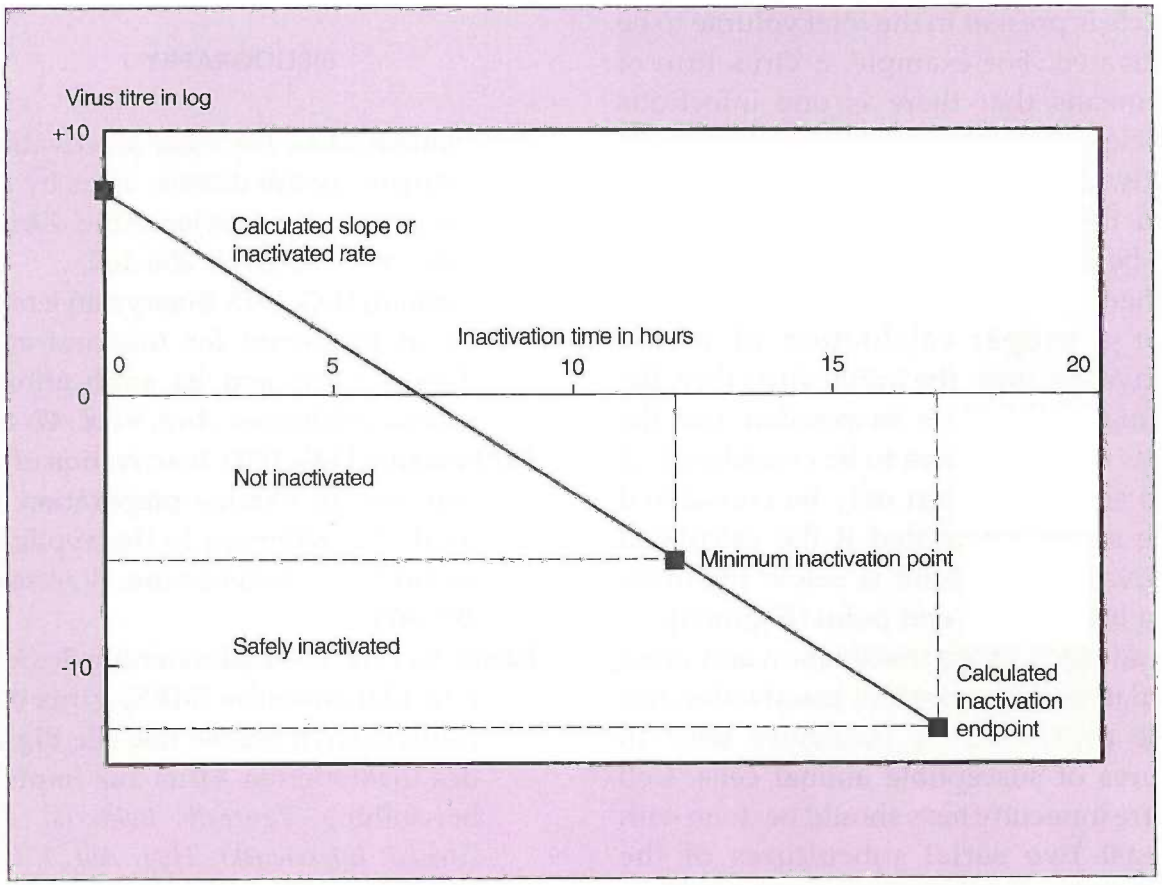

FIGURE 8
Control of inactivation of viruses with BEI

the inactivation curve, or the inactivation rate, is calculated by determination of the least squares regression line. With the known inactivation rate it is possible to calculate the minimum inactivation point and the minimum inactivation time required to reach that point.

The minimum inactivation point is defined as one log unit of virus infectivity below the point where one infectious particle is present in the total volume to be inactivated. For example, a virus titre of 10^{-3} means that there is one infectious particle in 1 000 ml or 1 litre. The minimum inactivation titre would therefore be 10^{-4}. From the known inactivation rate it can then be calculated at what time this titre is reached.

For a proper calculation of a safe inactivation time, the initial virus titre, the volume of the virus suspension and the inactivation rate have to be considered. A virus suspension can only be considered to be safely inactivated if the calculated inactivation end point is below the minimum inactivation end point (Figure 8).

In addition to the inactivation end point calculation the successful inactivation has to be controlled by innocuity tests in cultures of susceptible animal cells. Cell culture innocuity tests should be done with at least two serial subcultures of the maintenance medium at appropriate time intervals. The inactivated virus suspension can only be used for vaccine preparation after the inactivation end point calculation and the cell culture innocuity tests indicate complete inactivation.

OPERATIONAL CONSIDERATIONS

The inactivation of viral antigens should be carried out in an intermediate area that has a limited connection to the virus production area and no direct connection to the vaccine preparation area. The virulent antigen should only be inactivated in this area and can only be considered as inactivated on successful completion of the innocuity tests.

This means that the inactivation area has to be large enough to accommodate a laboratory for innocuity testing and storage facilities (cold rooms, tanks) for the antigen being tested. Only after the innocuity tests have shown that the antigen is properly inactivated can it be passed to the vaccine preparation area.

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Inactivation of antigens for veterinary vaccines (2)

Bacterial vaccines

H. de Ree

Vaccines for veterinary use are preparations containing antigenic substances which are applied for the purpose of inducing a specific and active immunity in animals against infectious diseases caused by bacteria and the toxins produced by bacteria.

In most cases veterinary bacterial vaccines are prepared with inactivated whole bacteria but in some vaccines the antigen is an immunogenic part of the bacterium, for example fimbriae or toxins. Toxins and whole bacteria have to be inactivated, otherwise the antigens will induce disease, and the inactivation of bacteria or toxins has to be examined carefully before the antigen can be included in a vaccine.

REQUIREMENTS FOR INACTIVATION

Requirements for the registration of veterinary vaccines are described in a European Pharmacopoeia Monograph (European Pharmacopoeia Commission, in press).

Inactivation kinetics

The inactivating agent and the inactivation procedure should be shown, under the conditions of vaccine manufacture, to inactivate the bacterium or the toxin. Adequate data on inactivation kinetics should be provided and normally the period of inactivation used in production should exceed the time shown to be adequate by the inactivation kinetics by at least 33 percent. The testing of the inactivation kinetics should carried out at least once.

Conditions for inactivation

Prior to inactivation, care must be taken to ensure a homogeneous suspension, free from particles that may not be penetrated by the inactivating agent.

Control of the inactivating agent

Appropriate tests must be carried out to demonstrate that the inactivant has been removed or depleted, or that any residues are safe, once the inactivation process has been completed. For example if formaldehyde is used as the inactivating agent then a test for free formaldehyde must be carried out.

Innocuity testing

A test for complete inactivation should be performed on the harvest immediately after termination of the inactivation procedure and, if applicable, the neutralization or removal of the inactivating agent. The test selected must be appropriate for the growth of the bacteria being used for vaccine production and must consist of at least two passages in production media or in media prescribed in the monograph specific to the vaccine being produced. No evidence of any live microorganism must be observed.

Bacterial toxoids are prepared from toxins by diminishing their toxicity to a very low level or by completely eliminating the toxic activity.

INACTIVATING AGENTS

Inactivation of bacterial suspensions is carried out with chemical agents such as formaldehyde or thiomersal. Sometimes

inactivation can also be performed with heat. Concentration of the inactivating agent and the temperature during the inactivation process must be determined and the presence of the inactivating agent in the ultimate vaccine (i.e. the final product) has also to be determined. The test for the determination of free formaldehyde can be made by an analytical method but also with a dipstick method, which is an easy and fast method. Not more than 0.05 percent m/V of free formaldehyde can be present in vaccines unless the higher concentration has been shown to be safe.

INACTIVATION OF BACTERIAL SUSPENSIONS

The method for inactivating bacterial suspensions can best be described by means of an example in which bacterium X is used as an antigen in an inactivated whole bacteria vaccine and the chemical inactivation is carried out with the inactivating substance Y.

For a general determination of the inactivation kinetics an inactivation curve can be established at the laboratory scale but this has to be confirmed later with a batch at the production scale.

After the bacterial fermentation has been completed the inactivant Y is added directly to the culture. Some substances also react with media components and it is therefore better to wash the bacteria first and to resuspend them in saline buffer before the agent Y is added.

After careful mixing to a homogenous suspension the bacterial culture is transferred to another vessel. This step is performed to circumvent the possibility that droplets of bacterial suspension on the inner part of the vessel, which do not come in contact with the inactivating agent, will reinfect the inactivated suspension.

The inactivation process is always carried out at a fixed temperature and the bacterial suspension has to be at that temperature before the start of inactivation. This is particularly necessary for very large volumes of production material since large volumes need a long time to reach the inactivation temperature.

Laboratory-scale inactivations will give an indication of the inactivation kinetics. Before and during the inactivation, samples are taken from the inactivation vessel and the live bacteria are counted by a validated method. The inactivation curve can be predicted for larger-scale productions by using the information from the laboratory batches. Reliable inactivation curves also have to be determined on large batches.

Differences between the kinetics for laboratory-scale batches and production batches are shown in Figure 9. At the laboratory scale it was demonstrated that inactivation of *Actinobacillus pleuropneumoniae* with a 0.1 percent thiomersal solution was complete within within days at 37°C. At the production scale (300 times larger than the laboratory scale), the inactivation was not complete after two days at 37°C and the inactivation time had to be prolonged by five more days.

With this inactivation curve the real inactivation time, including the 33 percent excess time, can be determined. Therefore, in the example shown in Figure 9, the total inactivation time has to be ten days.

The innocuity test to demonstrate complete inactivation has to allow optimal growth conditions for the agent X. For example, the inactivation of leptospira can be carried out with thiomersal but leptospira will not grow in the presence of small traces of thiomersal. Therefore, it is impossible to demonstrate that a sample taken directly from the inactivated suspension still contains live leptospira. The thiomersal has to be removed by a washing step or by diluting the sample so that the thiomersal is below the inhibiting concentration. Another method is to

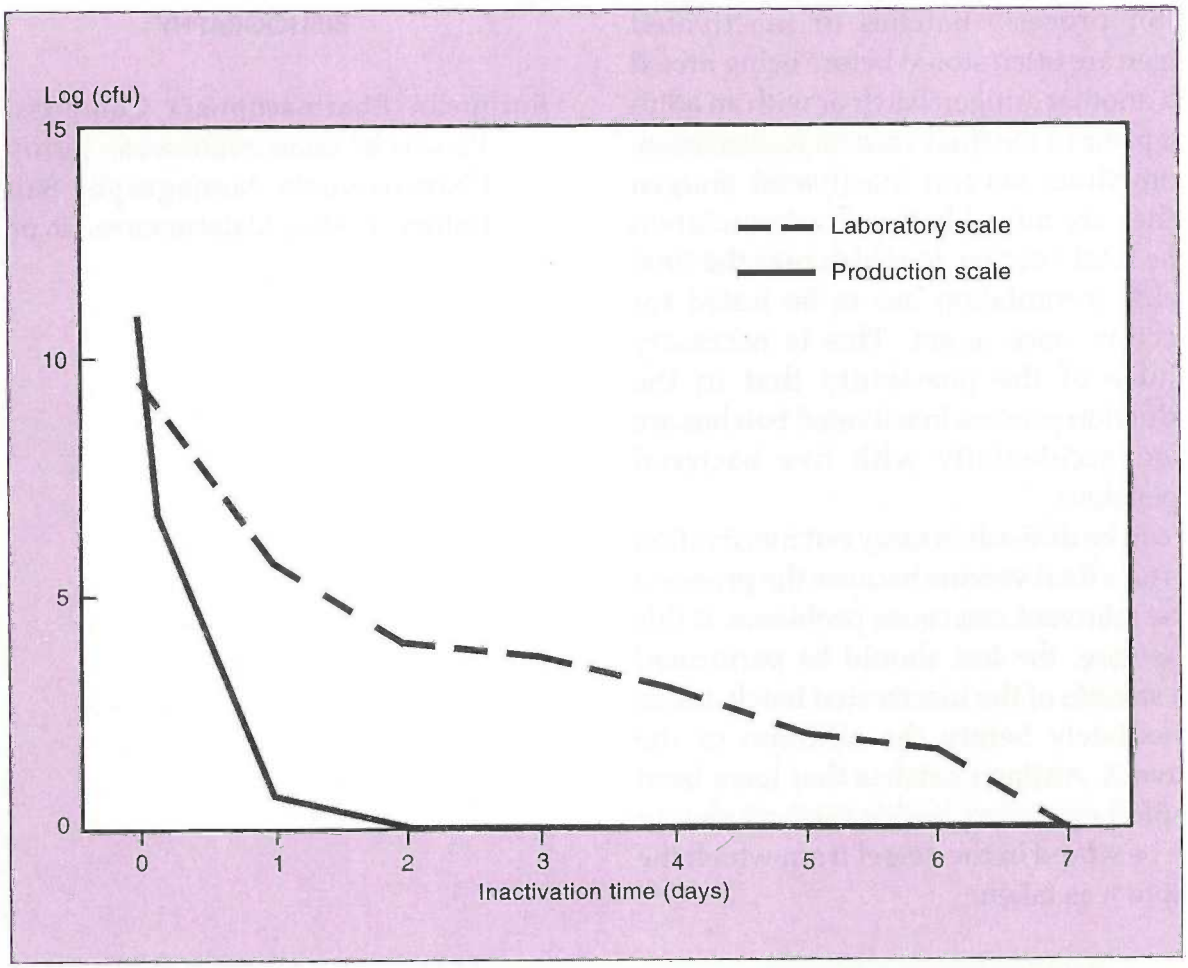

FIGURE 9
Inactivation curve of Actinobacillus pleuropneumoniae

neutralize the inactivating agent, for instance thiomersal can be inactivated with thioglycolate, but some bacteria are also sensitive to thioglycolate. This method cannot be used, for example, with leptospira. It is very important that the sensitivity of the test used is carefully determined.

The innocuity test has to be performed immediately after the prescribed inactivation process. Batches of inactivated antigen are often stored before being mixed with another antigen batch or with an adjuvant prior to the final vaccine formulation.

Sometimes several inactivated antigen batches are mixed before the formulation of the final vaccine, in which case the final vaccine formulation has to be tested for innocuity once again. This is necessary because of the possibility that in the production process inactivated batches are mixed accidentally with live bacterial suspensions.

It can be difficult to carry out inactivation tests on a final vaccine because the presence of the adjuvant can cause problems. If this is the case, the test should be performed on a sample of the inactivated batch, taken immediately before the addition of the adjuvant. Antigen batches that have been sampled according to this method should only be stored in the vessel from which the sample was taken.

INACTIVATION OF TOXINS

The requirements for bacterial toxins are the same as those described for the inactivation of bacterial suspensions. Absence of toxin activity in toxoid preparations has to be demonstrated in an animal model or in a cell test. The activity of the dermo-necrotic toxin of *Pasteurella multocida* can, for instance, be measured with the aid of Vero cells.

The irreversibility of the toxoiding process has to be determined by using the buffer for the final vaccine, without the adsorbent, and making a batch of purified toxin containing the same toxoid concentration as in the final vaccine. This batch is divided into two equal parts. One is kept at 5° ± 3°C and the other at 37°C for six weeks. Thereafter, both preparations are tested with a suitably sensitive assay for active toxin. Both must be free from any toxic activity.

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Adjuvants in veterinary vaccines

R. Bomford

Adjuvants are materials that are added to the antigens in a vaccine to stimulate an immune response. They can improve the performance of vaccines in various ways:

- They can increase the immune response induced by a given quantity of antigen. In general, live attenuated vaccines work well without an adjuvant, but inactivated or subunit vaccines, particularly highly purified recombinant antigens, usually require one.
- They can reduce the quantity of antigen needed to generate a protective immune response and may enable the vaccine to be made more cheaply. Fortunately some of the most widely used adjuvants, such as aluminium hydroxide gel ($Al(OH)_3$) or saponin, are not expensive and are easy to incorporate in vaccines. Whenever a decision is taken to include an adjuvant in a vaccine, it is worth titrating the antigen to see if a saving can be made.
- They can prolong the immune response so that only one administration of vaccine is required to protect the animal over its life span or season of exposure to the pathogen. This results in a saving of husbandry costs.
- They can be used to stimulate specific components of the immune response, such as particular antibody isotypes or cell-mediated immunity, which may be important for protection against the pathogen in question. In the majority of adjuvanted vaccines against bacterial and viral diseases that are currently used it is probably the circulating antibody that is important, but future vaccines against protozoal or multicellular parasites may need to induce mucosal or cell-mediated immunity to be effective and, as discussed in this chapter, there are adjuvant formulations that will achieve this.

The purpose of this chapter is to describe the procedures for using the adjuvants most widely included in veterinary vaccines and to provide a brief overview of new adjuvants that may find their way into future vaccines. For a more thorough review of the usage of adjuvants in particular veterinary vaccines, and of their mechanism of action, see Dalsgaard, Hilgers and Trouve (1990).

MINERAL GELS

Salts of aluminium, calcium, zinc, beryllium and other elements have been used as immunological adjuvants, but those most commonly found in human and veterinary vaccines are $Al(OH)_3$) and aluminium phosphate ($AlPO_4$) (Bunn, Nervig and Pemberton, 1986; Bomford, 1989a). $Al(OH)_3$ is used as an adjuvant for veterinary vaccines containing purified bacterial toxins, whole bacteria or inactivated viruses (Bunn, Nervig and Pemberton, 1986; Dalsgaard, Hilgers and Trouve, 1990) principally in large animals and also in some bacterial vaccines for poultry. However, the use of oil adjuvants for viral vaccines in poultry is increasing (Zanella and Marchi, 1982; McKercher, 1986). In some cases $Al(OH)_3$ may be used together with other adjuvants, for instance with saponin in foot-and-mouth disease (FMD) vaccines (Dalsgaard, 1978) or with an oil emulsion in sheep foot-rot vaccine (Thorley and Egerton, 1981).

There are two methods of preparing vaccines adjuvanted with $Al(OH)_3$. The first consists of mixing the antigen with a solution of alum, $KAl(SO_4)_2$, followed by the addition of NaOH, which will cause a precipitate of $Al(OH)_3$ to form according to the equation:

$$2KAl(SO_4)_2 + 6NaOH = 2Al(OH)_3 + 3Na_2SO4 + K_2SO_4$$

Vaccines prepared in this manner are referred to as alum-precipitated.This method can lead to somewhat variable results (Joo, 1973) and it is now more usual to add the antigen to pre-formed $AL(OH)_3$ gel, which can be prepared by adding 1 N NaOH to a 10 percent solution of alum, the precipitated gel being washed with saline or phosphate-buffered saline. Alternatively, ready-prepared $Al(OH)_3$ gels such as Alhydrogel (Superfos, Denmark) or AluGel-S (Serva, Germany) which have been tested for properties such as purity, stability and adsorptive capacity can be purchased.

The formulation of the vaccine is a simple process of ensuring a uniform mixing of the antigen and gel although care needs to be taken to ensure that the pH and electrolyte content of the mixing solution are suitable for optimal adsorption. Vaccines made by mixing the antigen with $Al(OH)_3$ are referred to as aluminium-adsorbed antigens.

$Al(OH)_3$ should be stored in containers made of aluminium, pyrex glass or inert plastic at 4°C. It should not be frozen, as this can be detrimental to the gel structure and adsorptive potential. It can be repeatedly sterilized by autoclaving (120°C for one hour), checking that the gel phase is always submerged beneath the aqueous phase to avoid dehydration changes. Because of the poor heat conductivity of suspensions of $Al(OH)_3$ gel, it is desirable to carry out sterilization in a stirred vessel.

The effectiveness of $Al(OH)_3$ as an adjuvant depends crucially on obtaining a good adsorption of the antigen to the gel (Lei, 1985; Seeber, White and Hem, 1991). Adsorption takes place by ionic interaction, under conditions in which the gel and the antigen carry opposite charges. At neutral, physiological pH $Al(OH)_3$ is positively charged and the majority of proteins are negatively charged, so adsorption is not usually a problem.

It is, however, essential to check that adsorption has occurred and this can be achieved by incubating a constant amount of antigen with varying amounts of gel overnight followed by centrifugation and measurement of the concentration of antigen remaining in the supernatant by an appropriate assay. If the majority of the antigen is not adsorbed by the quantity of gel it is intended to use in the vaccine, the following steps may be taken:

- First, check the electrolytes that are present in the antigen solution. Monovalent ions have no effect on adsorption at normal concentrations, but multivalent anions such as phosphates, sulphates or borates can interfere with adsorption.
- Second, test the adsorption of the antigen to $Al(OH)_3$ under different conditions of pH. The antigen and gel can be adjusted to different pH values prior to mixing, at pH intervals of 0.5 over a range compatible with the stability of the antigen and the pH requirements of the vaccine.
- Third, consider replacing $Al(OH)_3$ with $AlPO_4$, the latter being negatively charged at physiological pH and, hence, suitable for the adsorption of proteins which have a high isoelectric point and are positively charged at physiological pH (Seeber, White and Hem, 1991).

The quantity of $Al(OH)_3$ to be used in a vaccine is best determined by titration in

the target species. As a general guideline, the most important veterinary vaccines for cattle and pigs contain approximately 2 to 10 mg Al as the total aluminium content of a dose of vaccine. The dose-response curve of the adjuvant effect of $Al(OH)_3$ is bell-shaped (Hennessen, 1965), so that including too much adjuvant could reduce the stimulation of the response. It could also increase the risk of local reactions, although aluminium-adsorbed vaccines are generally well tolerated. The optimum dose of $Al(OH)_3$ does not change with the dose of antigen (Hennessen, 1965), so that once an optimum dose of $Al(OH)_3$ has been determined for one dose of antigen, it is possible to titrate the antigen keeping the dose of $Al(OH)_3$ constant.

SAPONIN

Saponins are natural products very widely distributed in the plant kingdom. Chemically they consist of a sterol or triterne ring structure, to which is attached one or more sugar chains. Since the ring structures are hydrophobic and the sugars are hydrophilic, saponins often possess a detergent-like activity and will lyse red blood cells.

In relation to vaccines the major application of saponin has been its use in the formulation and potentiation of FMD vaccines and some bacterial vaccines (Dalsgaard, 1978; Dalsgaard, Hilgers and Trouve, 1990). Saponin is a particularly good adjuvant for experimental vaccines against protozoal parasites (Bomford, 1989b).

The complex chemical structure of saponins gives great scope for heterogeneity, through changes in either the ring structures or the sugars. Not only are there differences among the saponins extracted from different plants, but even a single species of plant may produce a considerable variety (Price, Johnson and Fenwick, 1987).

It cannot be stressed too strongly that not all saponins are adjuvants and that a saponin intended for vaccine use should have a defined botanical origin and should be extracted in a reproducible manner. Saponins are produced in large amounts for the photographic industry or as foaming agents for soft drinks, and many preparations are sold as "saponin" without any indication of their origin, chemical nature or degree of purity.

The choice of a saponin is simplified by the fact that the saponins currently in use in veterinary vaccines are all extracted from one species, the bark of the South American tree *Quillaia saponaria*. Preparations of *Quillaia* saponin may differ in adjuvant activity according to the source or method of extraction, and the activity of new batches should be checked. A purified preparation containing a mixture of *Quillaia* saponins, named Quil A (Dalsgaard, 1978), is produced commercially as a standardized adjuvant for veterinary vaccines (Superfos, Denmark). Recently a defined component of *Quillaia* saponin (QS-21) which is adjuvant-active but lacks toxicity has been isolated (Kensil *et al.*, 1991). This is also produced commercially under the trade name Stimulon (Cambridge Biotech, United States) and is beginning to be used in veterinary vaccines.

Although it is preferable to use *Quillaia* saponin when available because of its proven efficacy in veterinary vaccines, saponins from other plants, *Gypsophila* and *Saponaria*, have shown adjuvant activity in experimental systems (Bomford *et al.*, 1992). These saponins are similar in structure to those of *Quillaia*, being hydrophilic with two sugar chains. Therefore, saponins other than those derived from *Quillaia*, might be considered for veterinary vaccines, provided their adjuvant activity is confirmed, their source is well defined and they are produced in a manner that yields a consistent product.

Saponins are supplied as lyophilized powders and are very stable at room temperature under dry conditions. They are water-soluble and may be sterilized by filtration.

The dose of saponin to be used in a vaccine is best titrated in the target species. The recommended dose of Quil A for cattle vaccines is 250 to 750 micrograms per dose of vaccine (Superfos, 1994), but possibly four to five times this amount of the less purified food-grade preparations of *Quillaia* saponins will be required.

Saponins are potentially toxic, causing both local and systemic side-effects, and new batches should be checked in the target species for effects such as lameness or loss of appetite. However, with a good-quality saponin preparation it is usually possible to find a dose that is adjuvant-active and non-toxic.

IMMUNOSTIMULATING COMPLEXES

The original idea behind immunostimulating complexes (ISCOMs) was to form a mixed micelle of surface antigen from enveloped viruses and saponin. This was achieved by splitting the virus particles with a detergent and centrifuging them into a solution of saponin which yielded cage-like structures around 35 nanometres in diameter (Morein *et al.*, 1987). However, it subsequently proved difficult to prepare ISCOMs from recombinant viral antigens, and it was realized that cholesterol and phospholipid, which are present in virus envelopes, are essential parts of the structure. Experimental protocols for preparing ISCOMs from lipid-free antigens now include these two lipids (Morein *et al.*, 1987; Dalsgaard, Hilgers and Trouve, 1990) and empty ISCOMs containing saponin and lipids but no antigen can also be made. Antigens require a lipophilic region in order to be incorporated into ISCOMs. This is available naturally in the surface proteins of enveloped viruses with their transmembrane region, and other antigens such as synthetic peptides can be adapted to associate with ISCOMs by conjugation to fatty acids.

The procedure for preparing ISCOMs from whole enveloped virus particles involves the purification of the virus on a lectin column, its solubilization with the detergent N-decanoyl-N-methyl-glycoside (MEGA-10) at a final concentration of 5 percent for two hours, the addition of Quil A to a final concentration of 0.1 percent and dialysis against 0.05 molar ammonium acetate buffer (Carlsson, Alenius and Sundquist, 1991). The ISCOMs should form spontaneously after the dialysis, but this needs to be checked by electron microscopy.

A great many ISCOM-based experimental veterinary vaccines have been prepared from enveloped viruses (Morein *et al.*, 1987; Dalsgaard, Hilgers and Trouve, 1990; Carlsson, Alenius and Sundquist, 1991), and two commercial ISCOM vaccines against equine influenza have been launched (Iscotec, Sweden; Equip, Pitman-More, United Kingdom). ISCOMs appear to be very effective for inducing mucosal immunity after oral immunization (Mowat and Donachie, 1991).

OIL EMULSIONS

The prototype oil-emulsion adjuvants are the so-called Freund's adjuvants, named after their inventor Julius Freund. The Freund's incomplete adjuvant (FIA) is a water-in-oil emulsion of mineral oil stabilized with the emulsifier mannitol mono-oleate. Freund's complete adjuvant (FCA) is the same emulsion with the addition of killed cells of mycobacteria in the oil phase. FCA is an extremely powerful adjuvant, but much too inflammatory for vaccine use. The adjuvant active component of mycobacteria has been identified and synthetic analogues of this,

the muramyl dipeptides, are being used in new adjuvant formulations.

A variety of veterinary vaccines against bacterial and viral diseases of cattle, pigs, sheep and poultry are adjuvanted with oil emulsions (McKercher, 1986; Dalsgaard, Hilgers and Trouve, 1990). These vaccines find a particular application in FMD vaccines for pigs (Anderson, Masters and Mowat, 1971; McKercher and Graves, 1977) and inactivated viral vaccines for poultry (Zanella and Marchi, 1982). Oil-emulsion adjuvants are particularly suitable for vaccines that will be required to give long-term protection. The antigen, being enclosed in the water droplets in the continuous phase of the oil is released slowly and provides a long-term stimulation of the immune system.

The technical factors that need to be taken into account for oil emulsions are the choices of oil and emulsifying agent, the proportions in which they are mixed together and with the antigen, the conditions of emulsification and the quality control of the finished emulsion.

The oil should be a light mineral oil of low viscosity containing hydrocarbons with a chain length of 12 to 30 atoms. Hydrocarbons of a lower chain length are more inflammatory (Gupta *et al.*, 1993). The following oils have all been found to be satisfactory in veterinary vaccines: Drakeol 6VR (Penrecco, United States), Marcol 52 and Marcol 82 (EXXON), Sontex 55 (Marathon Marco, United States), Vestan A50B (Fina) and Whitrex 307 (Mobil).

The usual emulsifying agent is a mannitol mono-oleate and it is preferable to use a product specially refined to be of injectable quality such as Arlacel A special (ICI, United States) or one of the range of Montanides (SEPPIC, France), rather than material intended for industrial use, which may contain impurities. These emulsifiers can be sterilized by autoclaving (120°C for 90 minutes) or filtration.

The ratio of oil to emulsifier is usually 9:1 by weight, although this can vary a little with different Montanides, and sterile ready-made mixtures of oil and emulsifying agent are available – the Montanide ISA adjuvants (SEPPIC, 1994). The proportion of the aqueous phase may be between 25 and 50 percent by weight, but the viscosity of the final emulsion increases with the water content.

A water-in-oil emulsion prepared with the emulsifying agents Span 85 (sorbitan triolate) and Tween 85 (polyoxyethylene 20 sorbitan triolate) has been described (Bokhout, van Gaalen and van der Heijden, 1981). The ratio of aqueous phase to mineral oil (Marcol 52) to emulsifying agents is 8:9:1 (by volume) and the Span 85 and Tween 85 are mixed in a ratio of 54:46 (by volume).

Industrial-scale emulsification can be carried out on a batch-to-batch basis in tanks of up to 300-litre capacity or by using two tanks of 300- to 1 000-litre capacity with an in-line homogenizer. Suitable homogenizers are sulplied by Silverson (Chesam, United Kingdom). The oil and the emulsifier are mixed in the tank at low speed and then the aqueous phase is added over about five minutes while the speed of homogenization is increased to around 4 000 rpm.

Homogenization is continued for about four minutes and the temptation to overhomogenize should be resisted because too much agitation may increase the viscosity of the emulsion, reduce its stability because of coalescence of water droplets and lead to overheating which could damage the antigen.

The following properties of the finished emulsion require to be checked:

- *The nature of the emulsion.* Is the emulsion water-in-oil, or oil-in-water? The simplest way of checking this is to place a small drop of the emulsion gently on the surface of cold water. If

the emulsion is water-in-oil the droplet should not spread out over the surface and should only break up, with difficulty, into large pieces when agitated. If, however, the emulsion is oil-in-water, the droplet will disperse immediately and mixing will produce a uniform milky suspension. The two types of emulsion can also be distinguished by their electrical conductivity, measured with a conductivimeter. The conductivity of water-in-oil emulsions is <5 microsiemens, whereas that of oil-in-water emulsions is >5 millisiemens.

- *The viscosity of the emulsion.* This can be measured by a rotating viscometer. The viscosity of the emulsion will vary according to the emulsifying agent and the proportion of the aqueous phase, but it should preferably not be higher than 400 centipoises from the point of view of ease of injection.
- *Stability of the emulsion.* The preparation should be stored at 4°C and checked regularly for changes in appearance or conductivity over a period of six months.

There is a tendency for oil-emulsion adjuvants to cause granulomatous reactions at the site of injection, the possible causes of which are reviewed in Gupta *et al.* (1993). The most important points relating to these reactions can be summarized as follows:

- The intensity of adverse reactions is to some extent dependent on the antigen that is used, crude bacterial antigens being worse than well-purified viral antigens.
- Different species show different susceptibilities to the adverse effects, poultry being particularly resistant.
- The probability of causing adverse reactions can be reduced by optimizing the emulsion constituents as described above.

NEW ADJUVANT SYSTEMS

The new adjuvant systems which are being developed for human recombinant vaccines against viruses are reviewed in Bomford (1992). Some of these are now undergoing clinical trials in humans in vaccines against AIDS (Bomford, 1994). It is worth mentioning a selection of these formulations here, because they often show a more powerful adjuvant activity than $Al(OH)_3$ with model antigens and, hence, might find their way into veterinary vaccines in the future.

Monophosphoryl lipid A (MPL)

The lipid A fraction of bacterial endotoxin is a powerful adjuvant but too toxic to include in vaccines. MPL is a less toxic derivative produced by removing one of the phosphate groups of lipid A isolated from remutants of *S. typhimurium* and *S. minnesota.* It works best as an adjuvant when incorporated into oil-in-water emulsion of squalene (a constituent of shark oil) together with trehalose dimycolate, a surface-active molecule of bacterial origin. This formulation is available commercially as the Ribi Adjuvant System (Ribi Immunochem, United States).

Pluronic polymers

Pluronics are block copolymers of polyoxyethylene and polyoxypropylene, and their adjuvant effect is also best expressed in oil-in-water emulsions of mineral oil or squalene stabilized with Tween. This adjuvant system is sold under the trade name Titermax (CyTRx, United States).

Muramyl dipeptide (MDP)

MDP, a synthetic molecule, is an analogue of the minimal structure of the peptidoglycan of the mycobacterial cell wall that mediates the adjuvant activity of mycobacteria in FCA. The original MDP

was too pyrogenic for vaccines, but less toxic analogues have been discovered. One of them, threonyl MDP, is formulated with a pluronic oil-in-water emulsion of squalene as the Syntex Adjuvant Formulation-1, SAF-1 (Syntex, United States). Another that is commercially available is glucosaminylmuramyldipeptide, GMDP, (GERBU, Germany).

ADJUVANTS FOR ANTIPARASITE VACCINES

Vaccines against protozoal and multicellular parasites would be of great value for animal husbandry in developing countries but have proved very difficult to develop, partly because of the need to identify the protective antigens and also because it is likely that immune mechanisms other than circulating antibody may play a role in protection. Adjuvants may help to solve the latter problem, and the special adjuvant requirements of parasite vaccines are reviewed in Bomford (1989b). As regards vaccines against protozoa, it is striking that saponin is a highly effective adjuvant, not only for experimental infections of mice but also for the protection of cattle against babesiosis. The situation with helminth vaccines is more complicated, in that there is evidence that the IgE antibody may be one component of immunity, the best adjuvant being $Al(OH)_3$ which preferentially stimulates IgE, at least in mice; although there is also evidence for the importance of cell-mediated immunity in other experimental systems, in which the best adjuvants are saponin or *Bacille Calmette Guerin* (BCG).

CONCLUSIONS

It is likely that the adjuvant requirements of the standard bacterial and inactivated viral veterinary vaccines will continue to be met with mineral gels, saponin and oil emulsions or combinations of these adjuvants. However, these do not solve all the problems of long-lasting immunity after a single vaccination and induction of mucosal and cell-mediated immunity. This may demand the introduction of new adjuvants or, alternatively, the problem may be solved with live recombinant vaccines which are expensive to develop but cheap to produce. An anti-rabies vaccine based on a vaccinia virus vector is already being used to protect foxes in Europe, and oral veterinary vaccines against enteric diseases with salmonella vectors are under development.

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Bottling, labelling and packaging of vaccines

V.A. Srinivasan, A.P. Kariath and P.B.V.P. Bangar Raju

Product presentation to attract customers has been receiving great attention in recent years and manufacturers of biological products all over the world are being forced to adopt new methods of packaging to make their products more competitive and attractive. However, bottling, labelling and packaging is a comprehensive subject which requires to be described from the point of view of mandatory requirements as well as customer satisfaction.

CONTAINERS

Containers are vital components in the drug and biological product dispensing system. An ideal container is chemically and physically inert and in no way affects the contents it holds. The dosage and presentation of products may vary and hence containers are required to be chosen according to the colour, quality and composition of the product, the storage conditions and the final state of the preparation, i.e. liquid, freeze-dried, etc. Vaccines are presented in either glass or plastic containers as single-dose or multi-dose packs depending on the requirements of the user.

Glass containers

Glass is principally silicon dioxide with varying amounts of metal oxides. The glass containers used for vaccine preparations should be tested for chemical inertness. Quality control tests can determine the amount of alkali released from a particular grade of glass under specified conditions to ensure there is no hydrolysis or leaching.

Glass containers suitable for packaging pharmaceutical preparations are classified as types 1, 2 and 3. They have the following properties:

- *Type 1* is borosilicate glass and is principally silicon dioxide and boric oxide. It has the least leachability and a low thermal coefficient of expansion. Type 1 glass is suitable for all products and should be the first choice whenever the use of glass is inevitable.
- *Type 2 and 3* glasses contain relatively high proportions of oxides of sodium and calcium which make them chemically less resistant.

Glass vials are convenient for the visual inspection of the contents but their main disadvantages are undue weight, larger space requirements and fragility.

Plastic containers

Various polymers of thermoplastics are used in the pharmaceutical industry. Polypropylene is the most common polymer used in the veterinary vaccine manufacturing industry. Owing to their thinner walls, plastics are not easily breakable and are less heavy than glass, making them economical to transport and preventing loss through breakages.

Controls

On receipt of containers from the manufacturer, an adequate number of samples should be taken and tested to confirm that they match the required specifications. To check vaccine containers for freedom from manufacturing defects, quality control tests

should be conducted for the determination of the dimensions of bottles, for example bottle and neck sizes, usable volume, wall thickness and general configuration compared to the manufacturer's specification. There should also be tests caried out to confirm the absence of leakage.

In addition to the above, long-term vaccine storage tests should be carried out to assess the ability of the containers to hold the contents in a safe and sterile condition over a defined period of time.

Every manufacturing plant should decide on the type of containers to be used for filling various products and should insist on the usage of only approved materials tested against specification. Proper care must be taken with regard to the storage of containers prior to use to avoid mix-ups, contamination and damage.

Closures

Containers are usually sealed with bungs which enable the withdrawal of contents by means of a hypodermic needle without loss of integrity. Bungs are made principally from either natural rubber (latex) or a synthetic polymer such as butyl or neoprene.

The ideal rubber closure should be chemically inert and no component of the vaccine should be absorbed or adsorbed. Butyl rubber bungs are most commonly used in the vaccine industry and are siliconized to make them non-reactive. Epoxy-coated butyl bungs are less prone to leaching and shedding of particulate material.

Rubber closures should be tested for elasticity, puncture resistance, hardness, porosity and inertness. Rubber bungs that exhibit the property of a low water vapour transmission rate are preferred for sealing freeze-dried products. Rubber bungs for freeze-dried products must have the appropriate slotting to ensure the effective removal of moisture during freeze-drying operations.

Aluminium seals

Aluminium is used to hold the rubber closure in place and provide a tamper-proof seal. As most vaccines have to be stored at low temperatures, the selection of aluminium seals is critical since they tend to corrode at low temperatures. Caps made of 0.2- or 0.3-mm thick aluminium sheet are preferable. It is possible to obtain single caps with a "tear-off" system at the centre of the cap or double caps with additional tamper-proof arrangements.

Seals should be subjected to thorough checking, especially of physical parameters such as thickness, diameter and height, so as to facilitate easy handling on automatic sealing machines. Where a variety of vaccines are manufactured, it is advantageous to have coloured seals to differentiate the various products. Such coloured seals must be tested for non-leaching of colour. Seals can be embossed with the name or logo of the manufacturer. As the seals are delicate, adequate care has to be taken to avoid damage while transporting from the supplier to the vaccine manufacturing unit.

BOTTLING REQUIREMENTS

The environment in which the product is processed has a direct effect on its quality. If the work area is contaminated or dusty, even products containing the best-quality ingredients can become unacceptable so the environment must meet the standards required for the operation. The work of the filling section consists of bottle preparation, filling and labelling. The ideal flow plan for these activities and the work areas needed follows:

- bottle washing area;
- preparation area;
- sterilization area;
- sterile bottle holding area;

- filling area;
- inspection area;
- quarantine area;
- labelling and packing area;
- storage area.

Bottle cleaning area

The operations in the bottle cleaning area comprise: bottle washing, bottle preparation, bung and seal washing and sterilization.

In the pharmaceutical industry, cleanliness of the final containers is of extreme importance to ensure safety of the product. Irrespective of whether they are plastic or glass, all containers should be washed free from dust particles and inert materials. The size of the work area depends on the type of bottle washing machine used and the number of containers to be handled. An area of 10 by 8 m is adequate for washing approximately 20 000 vials of 100-ml capacity per day.

Adjacent to the bottle cleaning area there should be a zone dedicated to the unpacking of cartons of new containers. Such an arrangement helps in keeping the other areas free of dust, which is often brought in with the cartons.

Washing of bottles. The washing of containers involves cleaning with powerful jets of detergent, hot water, distilled water and compressed air. Bottles are washed by means of a rotary washing machine or a tunnel washing machine.

The sequence used by a rotary washing machine is as follows: freshwater wash with detergent; first compressed air flush; hot water wash; second compressed air flush; distilled water wash; and third compressed air flush.

Tunnel washing machines work essentially in the same sequence as the rotary washing machine except that the movement is linear.

Bottles are cleaned on the outside by spraying hot water and distilled water through nozzles.

The loading and unloading of bottles are normally carried out manually by the operator. Automatic loading and unloading facilities are available for some machines, but these inevitably increase the initial purchase price.

The washed bottles are subsequently sent to the preparation area.

The washing area requires good exhaust systems, as heat and humidity are likely to be high.

Bung and seal washing area. Bungs come in direct contact with the final product and should therefore be subjected to thorough cleaning. The bungs are initially boiled with a mild detergent and then washed with hot water. To avoid particle shredding, agitation is carried out using compressed air. This is followed by rinsing with distilled water. The bungs are siliconized before being packed into stainless steel boxes for sterilization. The siliconization of rubber closures is carried out using commercially available silicone-release agents. Silicone, being an inert substance, does not react with the vaccine but forms a thin film that reduces the reaction of the vaccine with the rubber closures. Silicone also helps in the insertion of bungs into the necks of bottles either manually or with the use of an automatic bunging machine.

The aluminium seals are initially washed with tap water followed by filtered distilled water and dried. After inspection any defective seals are removed before the remainder are processed for sterilization.

Preparation area

In the preparation area washed bottles, bungs and seals are packed into suitable stainless steel containers for sterilization and are visually checked for cleanliness before packing. A sterilization indicator strip is placed on the lid of the container

and marked with details of the type of material, the date of sterilization, etc.

Sterilization area

The sterilization section should adjoin the aseptic filling area and be provided with facilities for sterilization. The sterilizers should have double doors and be installed across the wall so that sterilized containers can be unloaded directly into the aseptic area. Both dry-heat sterilizers and autoclaves are normally installed to cater for the sterilization requirements of glass and plastic containers, respectively. Materials sterilized by dry heat include glass containers and aluminium seals.

Polypropylene containers, bungs and also the garments used by operatives are sterilized by autoclaving. The autoclave cycle should include three vacuum pulsings before sterilization and vacuum drying after sterilization.

The sequence of autoclaving should be as follows: three consecutive cycles of vacuum pulsing and steam injection; sterilization at 134°C for five minutes; vacuum drying for 20 minutes; and sterile air injection.

The sequence of dry-heat sterilization should be as follows: drying for one hour at 80°C; sterilization for three hours at 160°C; and cooling with high-efficiency particulate adsorption- (HEPA-)filtered air for 30 minutes.

Sterile bottle holding area

The sterile bottle holding area is part of the sterile filling area. The size of this area depends on the filling rate and the number of containers filled per day. The doors on the sterile side of the autoclave and the dry-heat sterilizer open into this area where the sterilized materials are unloaded and allowed to cool and the area is supplied with HEPA-filtered air to ensure that the containers, bungs and aluminium seals remain sterile.

Filling area

The area at the centre of the filling operations is held under positive pressure with HEPA-filtered air to minimize the risk of contamination. Bottling machines are arranged sequentially under laminar airflow units to enable continuous flushing with HEPA-filtered air. The speed of the air should be maintained at 100 +/- 20 feet (approximately 6 m) per minute.

The construction of the filling area requires special attention. The walls, ceilings and floor should be smooth, impervious and constructed with waterproof material to enable regular cleaning. All light fixtures, utility service lines, etc. must be shrouded or recessed to reduce dust accumulation. Mechanical equipment and table tops should be covered with stainless steel. There should be no sinks inside the filling area. The passage of the product from the sterile filling area to the outside non-sterile area should be such that the conveyor belt stops at the perimeter of the sterile area and the passage of filled containers should be across a stationary surface.

The HEPA filters for the filling area should have an efficiency of 99.97 percent in removing particles of 0.3 microns or larger. Filters should be regularly validated for efficiency using appropriate tests. The room should be air-conditioned and humidity controlled (temperature of 22°C and relative humidity of 40 percent) to ensure the comfort of personnel and the avoidance of sweating while they work. The filling area should be monitored at regular intervals by the use of "settle" plates to assess microbial counts and any deviation from those normally observed should be properly dealt with. Entry to the filling area should be restricted to authorized persons only.

Personnel working in the filling area must be adequately trained, neat, orderly and reliable in order to ensure the safety of

operations. Facilities created to ensure product safety can be rendered virtually useless if personnel do not take sufficient care to observe aseptic precautions. Sterile, dirt-free garments and gloves must be worn before entry into the sterile areas. Garments should be such that operators feel comfortable when wearing them and the material should shed no fibres or particles.

Entry into the sterile filling area should be through a three-room entrance. In the first room, outside footwear should be removed. The hands should be thoroughly scrubbed with germicidal soap and dried. The second room should be the changing room where sterile garments and gloves are put on and the third room is for the disinfection of gloves before entering the filling area.

Filling may be carried out in the sterile filling area using one of the following devices:

- a semi-automatic filling machine with manual stoppering;
- an automatic filling machine with manual stoppering;
- an automatic filling machine with automatic stoppering.

The appropriate method can be chosen on the basis of the turnover of bottles required. Automatic stoppering machines are expensive.

Devices used for bottling. The filling of vaccines into containers is best done by using aseptic filling machines. Vaccines do not undergo terminal sterilization, so the product must be transferred from the bulk container or blending vessel into the final container with the utmost care during filling operations.

The delivery of vaccine into the final containers is achieved using a syringe that delivers a pre-set volume of liquid. The syringe is made of either glass or stainless steel. The delivery of liquid into the container is through a nozzle, the size of which varies according to the neck of the vial and the volume to be delivered.

The syringe is mounted on a motor-driven shaft and the piston slides backwards and forwards with the rotation of the shaft. The syringe assembly consists of two ball valves which control the inflow and outflow of the liquid. While retracting, the sliding piston causes a negative pressure which opens the inlet valve to allow the liquid to flow into the syringe the forward motion of the piston shuts the inlet valve and opens the outlet valve to deliver the liquid. Filling machines can have syringe assemblies delivering product into a fixed number of vials at one time. They have arrangements for holding vials at a point immediately below the nozzles. The nozzle slides down into the vial and during its upward motion delivers the liquid into the vial – by the time the nozzle has moved up to the vial neck delivery is complete. The bottles then move away on the conveyor belt as the transport-stopping mechanism is released, a fresh set of vials move into place and the filling process is repeated.

The movement of vials, nozzles and syringes and the delivery of the product are all synchronized by controls provided with the equipment. The tubing and syringes used for filling are easily dismountable for cleaning and sterilization. They are made of non-corrosive metals such as stainless steel (grade 316) and siliconized rubber. Some of the vaccines contain certain additives, such as aluminium hydroxide, and to prevent these from settling in the bulk container prior to filling, a magnetically coupled stirrer mechanism has to be provided.

Personnel. The key personnel involved in the various filling operations should have adequate qualifications recognized by the pharmaceutical and biologicals control

authority of the country concerned. All other personnel employed in the filling process should be adequately trained in aseptic operations and handling sterile products and should be familiar with all the various operations that are being carried out.

Inspection area

The containers filled with vaccine leave the filling area and are brought to the inspection area. Each container should be checked thoroughly for underfilling, improper sealing and the presence of extraneous material. Containers that do not meet requirements should be discarded.

Quarantine area

Vaccine containers should be stored separately in a designated area until all the mandatory tests are completed. After a batch has been cleared following quality control tests, the vaccine bottles are labelled and transferred to the commercial cold store prior to despatch.

Labelling and packing area

The term "labelling" describes all labels and other written or printed material placed on a container or on any package or wrapper in which it is enclosed. The labelling of vaccines (both bacterial and viral) must provide the user with all the information needed to ensure the safe and proper use of the product. Incorrect labelling is one of the most frequent causes of product misuse and product recall. Consequently, most government regulations insist that labels should carry the following information:

- The name of the preparation;
- the composition of the vaccine and the quantities of its individual ingredients;
- the name and percentage of bacteriostatic agent contained in the vaccine;
- the route of administration;
- storage conditions;
- the batch or lot number;
- the date of manufacture;
- the expiry date;
- the name and address of the manufacturer;
- the manufacturing licence number that has been given by an appropriate government authority.

The label should also indicate that the product is meant for veterinary use only, in accordance with the pharmaceutical and biological regulations of the appropriate authority of the country concerned.

Types of labels. Different types of labels are used in the pharmaceutical industry:

- paper labels of 70, 83 or 90 grams per square meter (gsm) on chromo-art paper;
- pre-gummed paper labels of 70, 83 or 90 gsm;
- pre-gummed polyvinyl chloride (PVC) labels.

Selection of appropriate labels is important to ensure their retention on containers during storage and transport at low temperature. Labels for each individual product should be stored separately to avoid their misapplication.

Controls. Each batch of labels should be checked and cleared for use on receipt according to the set procedures. The printing of labels in mass should be avoided. Excess labels bearing a particular batch number should be destroyed after completion of labelling of that batch and before commencement of labelling of subsequent batches.

Rejected labels should be counted and destroyed to avoid confusion. The area and equipment for the labelling operation should be thoroughly checked for any leftover labels from previous batches. The area where labels are stored should be accessible to authorized personnel only. Checks should be made on the number of

labels used against the number issued and proper reconciliation of the figures must be achieved. Any discrepancies should be investigated before the batch is released.

Packaging and distribution. The term "packaging" refers to all the materials and methods that help to make the product available to the customer in a suitable, convenient and safe manner.

The distribution of vaccine should essentially be carried out in such a way that vaccine is maintained at temperatures appropriate to avoiding the deterioration of its components. The transport of vaccine in tropical countries where atmospheric temperatures in summer may reach 45°C needs special attention. Packaging materials require careful selection and rigorous testing and will, in general, include such items as polystyrene boxes, corrugated boxes and cool packs.

Some of the important attributes of efficient packaging follow:

- The particular package used should ensure that the quality of the product is maintained until it reaches the customer.
- It should be pilfer- and tamper-proof.
- It should have adequate information about the product in addition to instructions regarding storage and transportation.

Vaccines are thermolabile and so require to be transported under conditions in which the temperature is maintained at between 2 and 8°C. The mode of transport should ensure there is no break in the cold chain. Transportation under cold chain conditions from supplier to point of application involves the use of insulated boxes with adequate cooling material to ensure the maintenance of required temperatures. Alternatively, refrigerated vans may be used to transport vaccines, but this method is very expensive and somewhat cumbersome.

The insulated boxes used for transport should be cheap and efficient. Expanded polystyrene (EPS) boxes, popularly called "thermocole boxes", are used for transporting vaccines and these are designed to meet the above requirements. The size and wall thickness of the box chosen depends on the number of vaccine containers to be transported in each box and on the anticipated transit time.

Figure 10 gives the specifications of an EPS box used for transporting ten bottles of vaccine packed in 300-ml containers for a period of 18 hours and maintained within the temperature range of 2 to 8°C.

EPS boxes should be manufactured with a density of polystyrene granules of 30 kilograms per cubic meter. The external dimensions of the box should be 424 mm by 355 mm by 233 mm. The box should be provided with two grooves on the lid to carry two cool packs as in Figure 10. The weight of each box should be 560 g +/- 5 g.

Cool packs. To maintain the required low temperature within the box for extended periods, cool packs are used. These contain brine frozen at -20°C or a gel containing a mixture of guar gum, gelatin, carboxy methyl cellulose and distilled water similarly frozen. These preparations act as a "heat sink" to maintain the temperature of the vaccine between 2 and 8°C. For transporting 20 bottles of vaccine, each of 300-ml capacity, two cool packs of the dimensions given in Figure 10 are required.

The containers that form the cool packs are made from high-density polyethylene as shown in Figure 11. They are designed to fit into the lids of EPS boxes and are embossed with the logo and name of the manufacturer. Cool packs should be able to withstand temperatures in the range of -20° to +40°C and a suitable cap should be provided to fit tightly on to the filling port of the cool pack body.

The heat absorbance capacity of cool

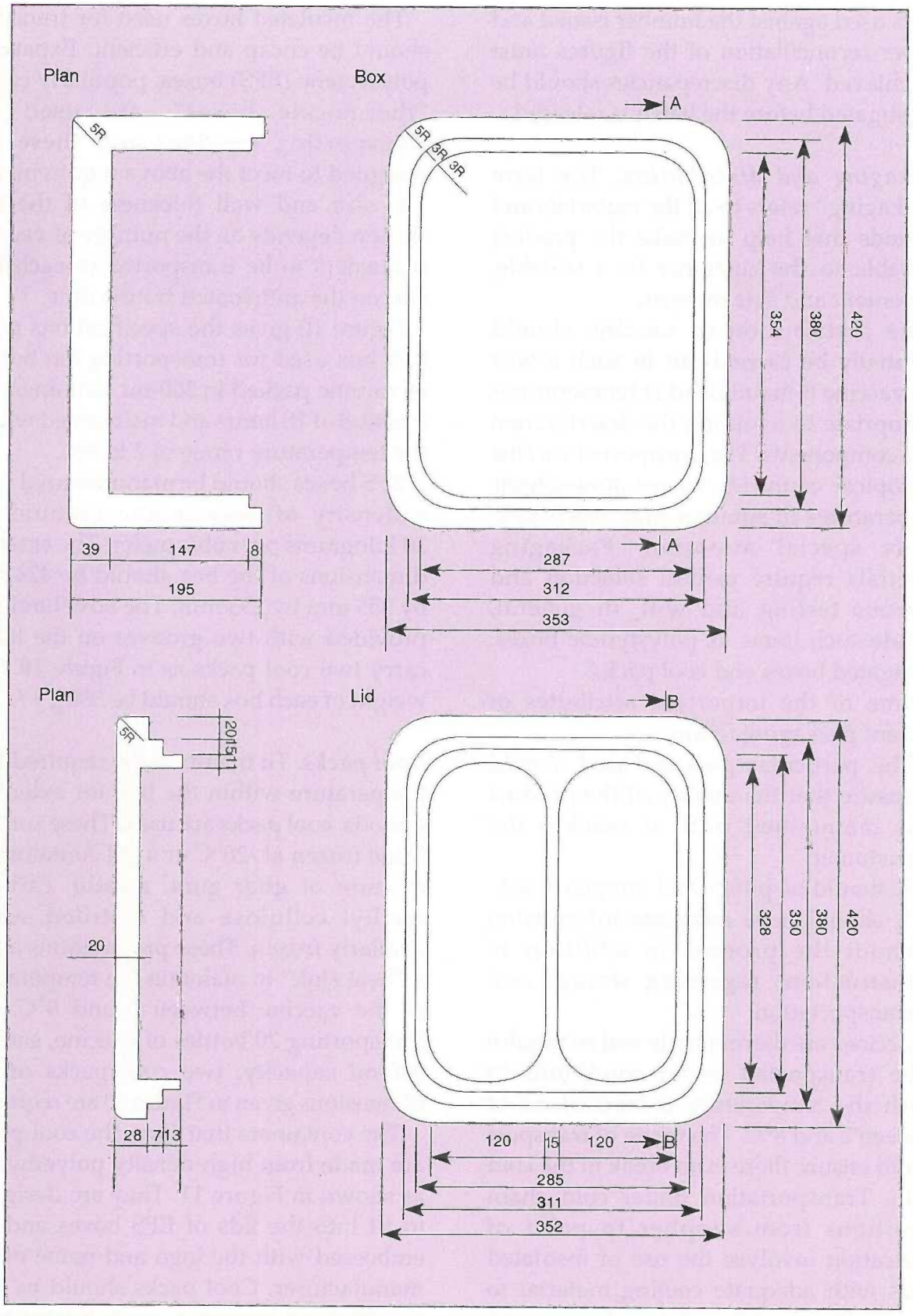

FIGURE 10
Dimensions of a polystyrene box and lid suitable for transporting 20 300-ml vaccine bottles. (Dimensions in mm)

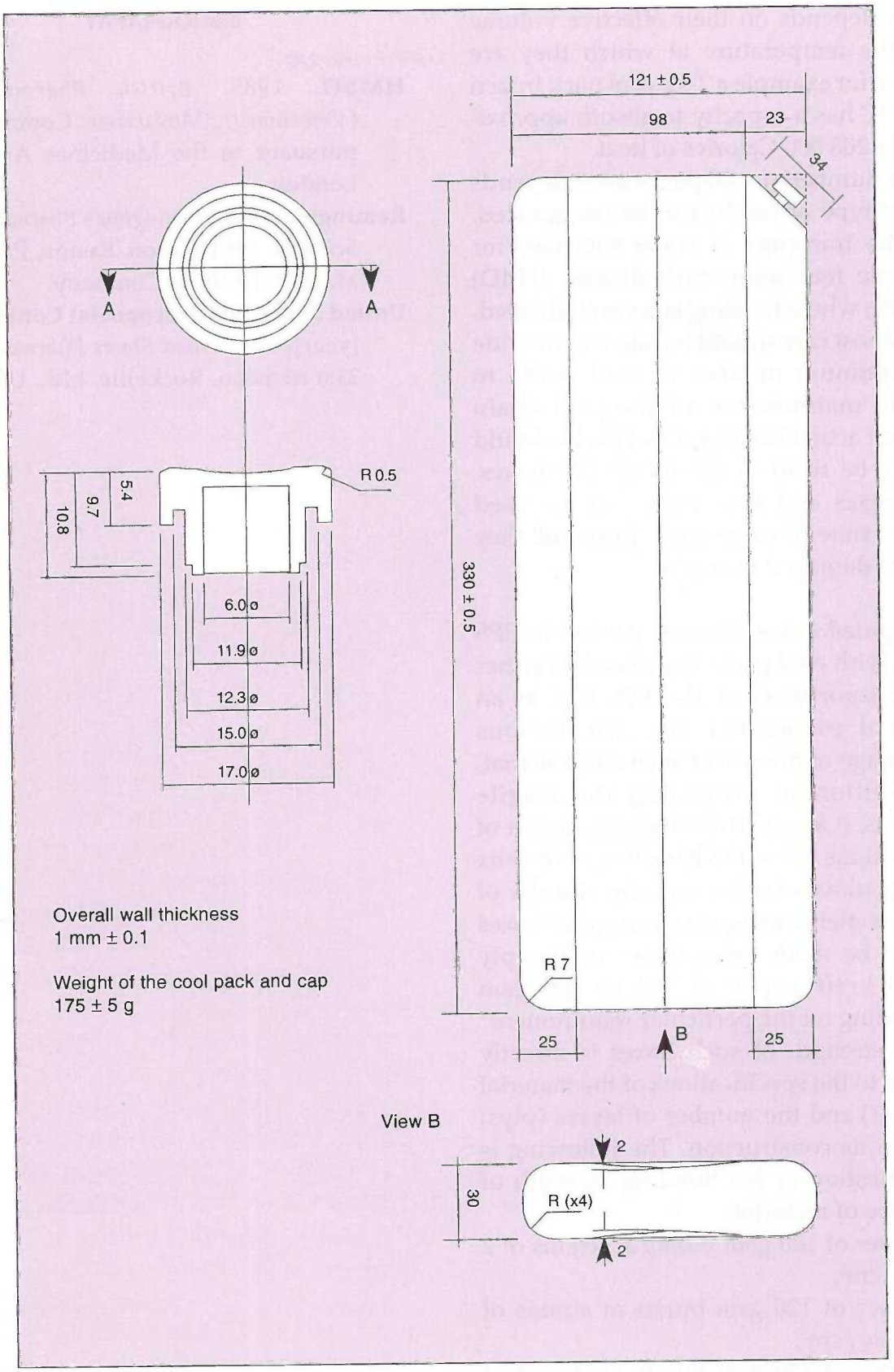

FIGURE 11
Dimensions of a cool pack

packs depends on their effective volume and the temperature at which they are frozen, for example a 2-kg cool pack frozen at -20°C has a capacity to absorb approximately 208 000 Calories of heat.

The number of cool packs used depends on the type of product to be transported. For the transport of some vaccines, for example foot-and-mouth disease (FMD) vaccine, where freezing is contraindicated, the utmost care should be taken to provide the optimum number of cool packs to ensure maintenance of the cold chain without actual freezing. Cool packs should ideally be fixed in the lid of EPS boxes. EPS boxes and cool packs can be used several times if necessary – provided they are not damaged in transit.

Corrugated boxes. Vaccine packed in EPS boxes with cool packs is protected further by transportation of the EPS box in an external corrugated box. An obvious advantage of the use of such a box is that, in addition to protecting the fragile contents, it also permits the attachment of instructions related to handling of the box during transportation and the address of the customer/consignee. Corrugated boxes should be made from three- or five-ply virgin kraft paper of 100 to 120 gsm depending on the particular requirement.

The strength of such boxes is directly related to the specifications of the material (its gsm) and the number of layers (plys) used in its construction. The following is an indication of the bursting strength of this type of material:

- paper of 100 gsm bursts at strains of 2 kg/cm^2;
- paper of 120 gsm bursts at strains of 2.5 kg/cm^2;
- paper of 150 gsm bursts at strains of 3.0 kg/cm^2.

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