

II. Laboratory Practices

A. Laboratory Techniques for Biohazard Control

Analysis of comprehensive surveys of laboratory acquired infections (1)* indicates that fewer than 20 percent of known infections can be attributed to a documented accidental exposure. Risk assessment studies, however, have demonstrated that aerosols are created by most laboratory manipulations that involve microorganisms (25). These results suggest that inhalation of undetected aerosols may have contributed to occupational illness among laboratory workers who have handled biohazardous materials (6,7).

Laboratory techniques for operations that have a high potential for creating aerosols are reviewed in this section. Four measures are recommended to decrease the hazard of exposure to aerosols.

MEASURES TO DECREASE HAZARDS OF AEROSOLS
FROM LABORATORY OPERATIONS

Operations that have the potential to create hazardous aerosols	Measures to decrease hazards from aerosols:			
	Avoid creating aerosol	Reduce extent of aerosol	Contain aerosol	Use personal respiratory protection
blowing the last drop of a liquid culture or chemical from a pipette .	x			
removing the cover from a Waring blender or dry chemical grinder shortly after completion of the blending or grinding operation		x	x	
removing the cap from a bottle of a liquid culture or suspension immediately after vigorous shaking; improper stoppering of volatile toxic substances		x	x	
grinding tissue with mortar and pestle or glass tissue grinder		x	x	
decanting the supernatant fluid after centrifugation.	x	x		
resuspending packed cells by shaking or mixing.		x	x	
inserting a hot wire loop in a culture.	x	x		
withdrawing a culture sample from a vaccine bottle	x	x		
opening a freeze dried preparation		x	x	
shaking and blending cultures and infected tissues in highspeed mixers .		x	x	
disrupting tissue cultures to release virus by shaking with glass beads		x	x	
streaking an inoculum on a rough agar surface	x			
sonic disruption of cells.		x	x	
inoculating mice via the intra nasal or other routes.		x	x	
harvesting cultures from embryonated eggs	x	x	x	
evacuating the atmosphere from a high vacuum steam sterilizer prior to sterilization of contaminated material	x	x		
removing cotton plugs from flasks and centrifuge tubes		x		
handling cages that held infected animals		x	x	x
handling large animals in open areas or in unventilated cages.		x		x

*The references for this section appear on page 39.

Measures that avoid the creation of an aerosol or reduce the extent of aerosol formation should be employed routinely. Measures that contain the aerosol are to be used when the research activity requires physical containment at levels of P2 and above.

1. Pipetting

Pipettes are basic scientific pieces, of equipment used throughout the world. They are used for volumetric measurement of fluids and for the transfer of these fluids from one container to another. The fluids that are handled are frequently hazardous in nature, containing infectious, toxic, corrosive or radioactive agents. A pipette can become a hazardous piece of equipment if improperly used. Safety pipetting techniques are required to reduce the potential for exposure to hazardous materials. The most common hazards associated with pipetting procedures involve the application of mouth suction. The causative event in more than 13 percent of all known laboratory accidents that resulted in infection was oral aspiration through a pipette. Contaminants can be transferred to the mouth if a contaminated finger is placed on the suction end of the pipette. There is also the danger of inhaling aerosols created in the handling of liquid suspensions when using unplugged pipettes, even if no liquid is drawn into the mouth. Additional hazards of exposure to aerosols are created by liquid dropping from a pipette to a work surface, by mixing cultures by alternate suction and blowing, by forceful ejection of an inoculum onto a culture dish, or by blowing out the last drop. It has been demonstrated by highspeed photography that an aerosol of approximately 15,000 droplets, most under ten micrometers, is produced when the last drop of fluid in the tip of the pipette is blown out with moderate force. While the aerosol hazard associated with pipetting procedures can only be reduced by use of safe techniques and of Biological Safety Cabinets, the potential hazards associated with oral ingestion can be eliminated by use of mechanical pipetting aids.

a. Safety Pipetting Aids

There are many commercially available safety pipetting aids

The parameters that deserve attention in a safety evaluation involve the ability of the pipetting aid:

- To perform without uncontrollable discharge (i.e., leakage) from the pipette or disposable tip.
- To function without contamination of the suction end of the pipette and, in turn, the pipetting aid, operator and vacuum lines.
- To transfer fluids without creating aerosols that spread surface contamination and result in inhalation and ingestion of hazardous substances.
- To be cleaned and sterilized in routine maintenance and preventive operations or following overt accidental contamination.

b. Safe practices governing the use of pipettes and pipetting aids.

- (1) Never use mouth pipetting. Always use some type of pipetting aid.
- (2) If working with biohazardous or toxic fluids, pipetting operations should be confined to a safety cabinet or hood.
- (3) Pipettes used for the pipetting of biohazardous or toxic materials always should be plugged with cotton (even when safety pipetting aids are used).
- (4) No biohazardous material should be prepared by bubbling expiratory air through a liquid with a pipette.
- (5) Biohazardous material should not be mixed by suction and expulsion through a pipette.
- (6) No biohazardous material should be forcibly expelled out of a pipette.
- (7) When pipettes are used, avoid accidentally dropping infectious cultures from the pipette. Place a disinfectant-soaked towel on the working surface and autoclave the towel after use.
- (8) Mark-to-mark pipettes are preferable to other types, since they do not require expulsion of the last drop.
- (9) Discharge from pipettes should be as close as possible to the fluid or agar level, or the contents should be allowed to run down the wall of the tube or bottle whenever possible, not dropped from a height.

(10) Contaminated pipettes should be placed horizontally in a pan containing enough suitable disinfectant to allow complete immersion. They should not be placed vertically in a cylinder.

(11) Discard pans for used pipettes are to be housed within the Biological Safety Cabinet.

(12) The pan and pipettes should be autoclaved as a unit. The replacement unit should be a clean pan with fresh disinfectant.

c. Microtitration, micropipettes and microdiluters

(1) Most serial dilutions made in the course of microtitrations use disposable trays with wells of 0.25 ml to 3.0 ml capacity. Dilutions are made using ultramicropipettes or microdiluters. Pipettes calibrated to deliver drops of 0.025 or 0.05 ml are available as disposable plastic or reusable, autoclavable polypropylene pipettes. Also available are specially designed microdiluters made of stainless steel and of a design for serial transfer and dilution of 0.025 or 0.05 ml volumes. The steel microdiluters are used in an automatic titrating machine or in a hand-held multidiluting device. From 8 to 12 serial dilutions are made simultaneously.

(2) Any ultramicropipette fitted with a bulb or other device that forcefully delivers material from the pipette creates a safety hazard if the last bit of fluid is blown from the pipette. Safer pipetting procedures are to deliver volumes mark-to-mark from the pipette or by draining to the pipette tip without splashing. Pipettes fitted with bulbs or plungers that forcefully eject liquids from the pipettes create aerosols, even if only microtitration is being performed.

(3) For safety in performing microtitrations with biohazardous materials, microdiluters that pick up a calibrated volume (0.025 or 0.05 ml), mix it with an equal volume in a well, and then transfer the calibrated volume to the next well are preferred. This system is relatively safe and accurate and performs multiple titrations simultaneously and swiftly.

(4) For intra-laboratory transport, the trays used for micro- titrations are placed in a closed container the outside of which is decontaminated. So contained, they can be safely placed in an incubator and then later returned to a safety cabinet for reading the results.

2. Syringes and Needles (8)

The hypodermic needle is a dangerous instrument. To lessen the chance of accidental injection, aerosol production or spills, its use should be avoided when alternate methods are available. For example, use a blunt needle or a cannula on the syringe for oral or intra nasal inoculations and never use a syringe and needle as a substitute for a pipette in making dilutions of dangerous fluids.

The following practices are recommended for use of the hypodermic needle and syringe when used for parenteral injections:

- Use the syringe and needle in a Biological Safety Cabinet only and avoid quick and unnecessary movements of the hand holding the syringe.
- Examine glass syringes for chips and cracks, and needles for barbs and plugs. This should be done prior to sterilization before use.
- Use needle locking (LuerLok^R type) syringes only, and be sure that the needle is locked securely into the barrel. A disposable syringe needle unit (where the needle is an integral part of the unit) is preferred.
- Wear surgical or other type rubber gloves for all manipulations with needles and syringes.
- Fill the syringe carefully to minimize air bubbles and frothing of the inoculum.
- Expel excess air, liquid and bubbles from a syringe vertically into a cotton pledget moistened with the proper disinfectant, or into a small bottle of sterile cotton.
- Do not use the syringe to expel forcefully a stream of infectious fluid into an open vial or tube for the purpose of mixing. Mixing with a syringe is condoned only if the tip of the needle is held below the surface of the fluid in the tube.
- If syringes are filled from test tubes, take care not to contaminate the hub of the needle, as this may result in transfer of infectious material to the fingers.
- When removing a syringe and needle from a rubber stoppered bottle, wrap the needle and stopper in a cotton pledget moistened with the proper disinfectant. If there is danger of the disinfectant contaminating sensitive experimental materials, a sterile dry pledget may be used and discarded immediately into disinfectant solution.

- Inoculate animals with the hand "behind" the needle to avoid punctures.
- Be sure the animal is properly restrained prior to the inoculation, and be on the alert for any unexpected movements of the animal.
- Before and after injection of an animal, swab the site of injection with a disinfectant.
- Discard syringes into a pan of disinfectant without removing the needle or manually replacing the protective needle sheath that is furnished with disposable hypodermic needles or syringe needle units. The syringe may be filled with disinfectant by immersing the needle and slowly withdrawing the plunger, and finally removing the plunger and placing it separately into the disinfectant. The filling action clears the needle and dilutes the contents of the syringe. Autoclave syringes and needles in the pan of disinfectant.
- In instances where the protective needle sheath must be replaced following use of a syringe (e.g., blood samples drawn for diagnostic purposes), forceps should always be used to minimize the possibility of exposure via accidental autoinoculation.
- Use separate pans of disinfectant for disposable and nondisposable syringes and needles to eliminate a sorting problem in the service area.
- Do not discard syringes and needles into pans containing pipettes or other glassware that must be sorted out from the syringes and needles.

3. Freeze-Drying Specimens

Specimens shell-frozen in ampoules are dried on a vacuum manifold or in a chamber-type drier at low negative pressure. If the glass neck of the ampoule is sealed off while it is still under vacuum, it may cause implosion either during sealing or later when the evacuated ampoule is being opened. To avoid this, after drying is completed and before sealing is done bring the pressure within the ampoule back to normal by gradually introducing dry nitrogen, avoiding turbulent disturbance of the dry product.

The narrow or constricted neck of ampoules is contaminated if the specimen is allowed to run down the wall of the neck during filling. Subsequently, when the ampoule is sealed with a torch, the dried material on the wall becomes charred or partially decomposed; residues of this material may adversely affect the dried material when it is reconstituted. To avoid this, a syringe with a long cannula or a Pasteur-type pipette should be used to fill the vial. Do not allow the delivery end of the cannula or pipette to touch the neck of the vial.

All ampoules used for freeze-drying of cultures, toxins or other hazardous materials should be fabricated in Pyrex-type glass. This glass requires a high-temperature torch using an air-gas or oxygen-gas mixture for sealing. These hard glass ampoules are much less apt to form glass bubbles that burst inwardly during sealing under vacuum than the soft glass ampoules and, of course, are more resistant to breakage from heat-shock, handling and storage.

The freeze-drier tubes, manifold, condenser pump and other internal parts will be contaminated after use (9). When dry infectious organisms are being prepared, a significant biohazard exists. Whether infectious or noninfectious living organisms, or toxic or nontoxic nonliving agents are being treated, if purity of product is a major concern, decontamination should be carefully considered. Protection by use of air filters should be given to vacuum lines and oil pumps. Freon-ethylene oxide gas, steam sterilizing, and liquid decontaminants should be employed as

appropriate, depending on the biological or chemical agent in use and the composition of mechanical parts of the drier.

The chamber type freeze driers allow the use of automatic plugging and screw capping of vials and also the use of tray drying in place of drying in ampoules or vials. The biohazards and chances of product contamination still exist and must be considered as with manifold-drying systems.

Protection against contamination of vacuum lines and pumps should be provided by appropriate filter systems.

The filling of ampoules and vials with infectious specimens and subsequent freeze-drying and sealing or closing of glass ampoules and vials in the preparation of dry infectious specimens should be done in a Biological Safety Cabinet. The same is true for preparation of ampoules and vials of liquid specimens not subjected to freeze-drying.

4. Opening Culture Plates, Tubes, Bottles, and Ampoules; Inoculating and Harvesting Cultures.

In the absence of definite accidents or obvious spillage, it is not certain that opening of plates, tubes and bottles of other microorganisms has caused laboratory infection. However, it is probable that among the highly infective agents some infections have occurred by this means (9). Particular care is required when opening plates, tubes, or bottles containing fungi, for this operation may release a large number of spores. Such cultures should be manipulated in a Biological Safety Cabinet (10,11).

To assure a homogenous suspension that will provide a representative sample, liquid cultures are agitated before a sample is taken. Vigorous shaking will create a heavy aerosol. A swirling action will generally create a homogenous suspension with a minimum of aerosol. When a liquid culture is resuspended, a few minutes should elapse prior to opening the container to reduce the aerosol.

The insertion of a sterile, hot wire loop or needle into a liquid or slant culture can cause spattering and release of an aerosol. To minimize the aerosol production, the loop should be allowed to cool in the air or be cooled by touching it to the inside of the container or to the agar surface where no growth is evident prior to contact with the culture or colony. Following use of the inoculating loop or needle, it is preferable to sterilize the instrument in an electric or gas incinerator specifically designed for this purpose rather than heating in an open flame. These small incinerators have a shield to contain any material that may spatter from the loop. Disposable inoculating loops are available commercially. Rather than decontaminating them immediately after use with heat, they are discarded first into a disinfectant solution.

The practice of streaking an inoculum on rough agar results in aerosol production, created by the vibrating loop or needle. This generally does not occur if the operation is performed on smooth agar. It is good safety practice to discard all rough agar poured plates that are intended for streaking purposes with a wire loop.

Water of syneresis in petri dish cultures usually contains viable microorganisms and forms a film between the rim and lid of the inverted plate. Aerosols are dispersed when this film is broken by opening the plate. Vented plastic petri dishes where the lid touches the rim at only three points are less likely to offer this hazard (12). The risk may also be minimized by using properly dried plates, but even these (when incubated anaerobically) are likely to be wet after removal from an anaerobic jar. Filter papers fitted into the lids reduce, but do not prevent, dispersal. If plates are obviously wet, they should be opened in the Biological Safety Cabinet (13).

Less obvious is the release of aerosols when screw-capped bottles or plugged tubes are opened. This happens when a film of contaminated liquid which may collect between the rim and the liner, is broken during removal of the closure (6). The practice of removing cotton plugs or other closures from flasks, bottles, centrifuge tubes, etc., immediately following shaking or centrifugation can generate aerosols and cause environmental contamination. The technique of shaking tissue cultures with glass beads to release viruses can create a virus-laden aerosol. Removal of wet closures, which can occur if the flask or centrifuge tube is not held in an upright position, is also hazardous. In addition, when using the centrifuge, there may be a small amount of foaming and the closures may become slightly moistened. Because of these possibilities, it is good safety practice to open all liquid cultures of infectious or hazardous material in a Biological Safety Cabinet wearing gloves and a long-sleeved laboratory garment.

Dried, infectious culture material may also collect at or near the rim or neck of culture tubes and may be dispersed into the air when disturbed (14). Containers of dry powdered hazardous materials should be opened only in a Biological Safety Cabinet (10, 15). When a sealed ampoule containing a lyophilized or liquid culture is opened, an aerosol may be created. Aerosol creation should be prevented or minimized, and opening of ampoules should be done in safety cabinets.

When recovering the contents of an ampoule, care should be taken not to cut the gloves or hands or disperse broken glass into the eyes, face, or laboratory environment. In addition, the biological product itself should not be contaminated with foreign organisms or with disinfectants. To accomplish this work in a safety cabinet and wear gloves. Nick the ampoule with a file near the neck. Wrap the ampoule in disinfectant wetted cotton. Snap the ampoule open at the nick, being sure to hold the ampoule upright. Alternatively, at the file mark on the neck of the ampoule, apply a hot wire or rod to develop a crack. Then, wrap the ampoule in disinfectant wetted cotton, and snap it open. Discard cotton and ampoule tip into disinfectant. The contents of the ampoule are reconstituted by slowly adding fluid to avoid aerosolizing the dried material. Mix contents without bubbling, and withdraw it into a fresh container (13). Some researchers may desire to use commercially available ampoules pres cored for easy opening. However, there is the possibility to consider that this may weaken the ampoule and cause it to break during handling and storage. Ampoules of liquid cultures are opened in a similar way.

Harvesting cultures from embryonated eggs is a hazardous procedure and leads to heavy surface contamination of the egg trays, shells, the environment, and the hands of the operator. It is essential that operations of this type be conducted in a Biological Safety Cabinet. A suitable disinfectant should be at hand and used frequently.

5. Centrifuging

Centrifugation presents two serious hazards: mechanical failure and dispersion of aerosols. A mechanical failure, such as a broken drive shaft, a faulty bearing, or a disintegrate rotor, can produce not only aerosols but also hazardous fragments moving at great velocity. These fragments, if they escape the protective bowl of the centrifuge, could produce traumatic injury to personnel. A well functioning centrifuge, however, is still capable of producing hazardous aerosols of biological material or chemicals if improperly used or in the absence of good laboratory practices. Mechanical failure can be minimized by meticulous observance of the manufacturers' instructions, and aerosols can be avoided by observing sound laboratory practices and use of appropriate centrifuge safety equipment or Biological Safety Cabinets (16-19).

Although accidents from improper use of centrifuges and equipment associated therewith are far less frequent than with pipettes or syringes and needles, when they do occur aerosols usually are created, and the possibility of causing multiple exposures is considerably greater. This conclusion is borne out by data presented in the following table from the proceedings of a symposium held on centrifuge biohazards in 1973 (20) .

INFECTION OR HYPERSENSITIVITY FROM CENTRIFUGING MICROBIAL MATERIAL

Disease	Comment	Persons Affected
Brucellosis	Aerosol spread from basement to 3rd floor	94
Glanders	Tube broke	3 (2 fatal)
Plague	Fluid spun off lip of intact centrifuge tube	1
Q fever	"Use of a centrifuge"	60
Q fever	"Spread from 1st to 3rd floor*"	47
Q fever "	Throughout the building, "Centrifuging or grinding tissue"	15
Tuberculosis	Broken tube and a hole in trunnion cup .	2
Tularemia	"Principally the pipetting and centrifugation"	1
Tularemia	Centrifuging	1
Western equine encephalitis	"Virus was thrown out"	1
Allergy attacks	Preparing antigens in a Sharples centrifuge	7
Allergy attacks	Killed M. tuberculosis	1

*Waring blender used, and "high speed" centrifugation of formalinized suspensions and subsequent resuspension of sediments."

Activities, such as filling centrifuge tubes, removing cotton plugs and rubber caps from tubes after centrifugation, removing the supernatant and resuspending the cells, are capable of releasing aerosols into the environment. The greatest hazard associated with centrifuging biohazardous materials is created when a centrifuge tube breaks. When tubes break or crack and a fluid containing microorganisms remains in the cup under centrifugal force, relatively few organisms are released into the air compared to breakage that releases the fluid into the centrifuge chamber.

a. Safety Procedures Applicable to All Centrifuging

A safety centrifuge cabinet or safety centrifuge trunnion cup should be used when centrifuging hazardous or infectious substances. When bench type centrifuges are used in a Biological Safety Cabinet, the glove panel should be in place with the gloves in place or with the ports covered. The centrifuge operation creates air currents that may cause the escape of agent from an open cabinet (17,27,28).

Centrifuge tubes and trunnion cups should be filled and opened in a Biological Safety Cabinet (29). If centrifugation is to be performed outside the cabinet, the safety trunnion cup should be used. After it is filled and sealed, it should be considered potentially contaminated and should be wiped with a cloth soaked in disinfectant or passed through a disinfectant dunk bath. Since some disinfectants are corrosive to centrifuge cups and heads, a rinse of the cup with clean water is desirable after an appropriate contact time has elapsed.

In some situations, in the absence of "O" ring sealed trunnion cup caps, specimens can be enclosed in sealed plastic bags before centrifugation (24). In the event of breakage, however, the plastic bag is likely to be ruptured. Thus, this technique normally only prevents the escape of organisms that contaminated the outside of the cup.

Before centrifuging, eliminate tubes with cracks and chipped rims, inspect the inside of the trunnion cup and correct rough walls caused by erosion or adhering matter, and carefully remove bits of glass and other debris from the rubber cushion (22,25).

A disinfectant should be added between the tube and trunnion cup to disinfect the materials in case of accidental breakage. This practice also provides an excellent cushion against shocks that might otherwise break the tube (22,25). Care must be taken, however, not to contaminate the culture material with the disinfectant. It must be recognized also that the disinfectant may not completely inactivate the infectious material when the tube breaks because of the dilution of the disinfectant and the high concentration and packing of cells.

Avoid pouring the supernatant material from centrifuge tubes. If you must do so, wipe off the outer rim with a disinfectant afterwards; otherwise, in a subsequent step, biohazardous fluid may be spun off as droplets that form an aerosol (22,25). Use of a vacuum system with appropriate inline safety reservoirs and filters is preferable to pouring from centrifuge tubes or bottles.

If the sediment is packed infectious microorganisms or other hazardous material and must be resuspended in order to minimize the amount of aerosol created, it is better to use a swirling, rotary motion rather than shaking. If vigorous shaking is essential to suspend the material or achieve homogeneity, a few minutes should elapse before opening the container to allow the aerosol to settle. Shaking always contaminates the closure; thus, there is the added hazard of liquids dropping from the closure or running down the outside of the container. A Biological Safety Cabinet with gloves in place may be required to assure safety to the laboratory worker when performing some of these operations.

Avoid filling the centrifuge tube to the point that the rim, cap, or cotton plug becomes wet with culture (22,25).

Screw caps or caps that fit over the rim outside the centrifuge tube are safer than plugin closures. Some fluid usually collects

between a plugin closure and the rim of the tube. Even screw capped bottles are not without risk, however; if the rim is soiled and sealed imperfectly, some fluid will escape down the outside of the tube (14).

Aluminum foil should not be used to cap centrifuge tubes containing toxic or infectious materials because these light-weight caps often become detached or ruptured during handling and centrifuging (14).

The balancing of buckets and trunnion cups is often improperly performed. Care must be taken to ensure that matched sets of trunnions, buckets and plastic inserts do not become mixed. If the components are not inscribed with their weights by the manufacturer, colored stains can be applied for identification to avoid confusion. When the tubes are balanced, the buckets, trunnions and inserts, including any disinfectant solution or water added for balancing, should be included in the procedure. The basic concern is that the centers of gravity of the tubes are equidistant from the axis of rotation. To illustrate the importance of this, two identical tubes containing 20 g of mercury and 20 g of water, respectively, will balance perfectly on the scales; however, their performance in motion is totally different, leading to violent vibration with all its attendant hazards (26).

b. Older Type and Small Portable Centrifuges

Older type centrifuges that do not have aerosol tight chambers have been shown to allow the escape of aerosol created from various sources:

- biohazardous fluid remaining on the lip of the tube after decanting the supernatant fluid
- leakage from a tube in an angle head centrifuge resulting from overfilling a tube and placing aslant in the centrifuge
- leakage from nonrigid tubes that distort under centrifugal forces, or
- fluid trapped in the threads of screw caps.

Safety trunnion cups should be used to prevent escape of aerosol in the event the primary culture container held in the cup should break or in any other manner allow the release of agent into the cup. The handling of the culture, the filling of centrifuge tubes and placing them in the safety trunnion cups should be done in a Biological Safety Cabinet. The outside of the trunnion cup should be decontaminated before the cup is removed for centrifuging. Subsequently, the cup should be returned to and opened in a Biological Safety Cabinet. Where applicable, the centrifuge itself should be placed in the cabinet, and, if need be, a cabinet should be specifically constructed for the centrifuge.

Small portable, "Clinical" centrifuges have been shown to be hazardous (27). The microhematocrit centrifuge, in particular, has been shown to produce aerosols. A frequent practice is to centrifuge blood samples in tubes without closures or to use cotton plugs secured in the tubes by means of tape or pins. It should be recognized that some tissue specimens contain viable infectious microorganisms, particularly hepatitis virus, and that open tubes, contaminated closures, and release of aerosols from blood samples and tissue suspensions can be hazardous to laboratory personnel.

c. Sharples Centrifuges

Using the Sharples centrifuge with infectious or hazardous materials poses both engineering design and safety problems. The Sharples centrifuge is driven by a steam or air turbine, requires refrigeration around the bowl, and is equipped with feed and effluent lines. It has a continuous feed that could involve large volumes of liquid material, depending on the amount of solids in the material to be handled and the type of bowl. The centrifuge generates a massive aerosol that is almost impossible to contain within the instrument even with a hermetically sealed bowl (19). For these reasons, a ventilated safety cabinet is necessary to enclose the centrifuge. It may be desired to accommodate the material to be centrifuged and the effluent in the cabinet or handle it by means of connectors through the walls of the cabinet. If the rotor must be transferred to another cabinet

after use, it should be passed through a dunk bath, wrapped in a disinfectant-soaked towel, or placed in another container, the outside of which is decontaminated. Decontamination of the centrifuge bowl, lines, and surrounding cabinet can be accomplished by liquid disinfectants, for aldehyde vapor or ethylene oxide, followed by additional cleaning and rinsing. The rotor can be steam sterilized.

d. High-Speed Centrifuges

Centrifugation at high speeds presents additional hazards because of the higher stresses and forces applied to components of the system. In addition to the recommended practices listed above, precautions should be taken to filter the air exhausted from the vacuum lines, to avoid metal fatigue resulting in disintegration of rotors, and to apply proper techniques in cleaning, handling, and using centrifuge components. Some of these precautions are discussed briefly below.

In high-speed centrifuges, the chamber is connected to a vacuum pump. If there is a breakage or accidental dispersion of infected particles, the pump and the oil in it will become contaminated. A HEPA filter should be placed between the centrifuge and the pump (14).

High-speed rotor heads are prone to metal fatigue, and, where there is a chance that they may be used on more than one machine, each rotor should be accompanied by its own log book indicating the number of hours run at top or de-rated speeds. Failure to observe this precaution can result in dangerous and expensive disintegration. Frequent inspection, cleaning, and drying are important to ensure absence of corrosion or other damage that may lead to the development of cracks. If the rotor is treated with disinfectant, it should be rinsed with clean water and dried as soon as the disinfectant has adequately decontaminated the rotor. Rubber "O" rings and tube closures must be examined for deterioration and be kept lubricated with the material recommended by the makers. Where tubes of different materials are provided (e.g.; celluloid, polypropylene, stainless steel), care must be taken that the tube closures designed specifically for the type of tube in use are employed. These caps are often similar in

appearance, but are prone to leakage if applied to tubes of the wrong material. When properly designed tubes and rotors are well maintained and handled, leaking should never occur (26).

Cleaning and disinfection of tubes, rotors and other components require considerable care. It is unfortunate that no single process is suitable for all items, and the various manufacturers' recommendations must be followed meticulously if fatigue, distortion and corrosion are to be avoided. This is not the place to catalogue recommended methods, but one less well appreciated fact is worthy of mention. Celluloid (cellulose nitrate) centrifuge tubes are not only highly flammable and prone to shrinkage with age and distortion on boiling, but also can be highly explosive in an autoclave (26).

e. Large-Scale Zonal Centrifuges

Zonal centrifuges have been developed to process relatively large volumes, 5 to 150 liters, of material. The pumps, valves, seals, feed lines, connectors, and vacuum and cooling systems, associated with these centrifuges, as well as the large volumes processed at high speeds, create the potential for leakage and generation of hazardous aerosols leading to the contamination both of the environment and of the operating personnel. The following areas have been identified as the principal sources of potential leakage: the centrifuge lip and face seals, the coolant system, the turbine exhaust air, various lines and connectors, the feed system, f action collection, and during decontamination. In addition, the possibility of spills occurring during loading, unloading, sample collection, decontamination, and other procedures must be recognized (28,29).

The several seals in the equipment pose the greatest potential for escape of hazardous material because inherent to the system is the necessity for pressurizing the process fluid to obtain flow through the rotor; in addition, leaks, may occur because of the large centrifugal forces exerted at all points in the rotating component. Procedural hazards identified include: (a) the danger of snagging or rupturing one of the numerous lines (influent, effluent, rotor by-pass, etc.), particularly when

hemostats are used as clamps;(b) undetected over pressurization of lines resulting in a rupture of a line or failure of a connection because flow was obstructed by bubbles caught in the system; c) manual making and breaking of connections during the centrifuge operation; (d) inadequate precautions in handling gradient fractions containing very high concentrations of the purified material; and (e) incomplete or ineffective decontamination procedures of the rotor, feed lines and other components of the equipment before disassembly and cleanup (30-32).

Zonal centrifuges that employ B rotor systems can be readily adapted under Class I cabinets. Some laboratories have attached a fume hood onto the centrifuge and have installed a HEPA filter in the exhaust duct from the hood. This type of arrangement permits utilization of the hood for most activities associated with the centrifugation process. It is suggested that a sink be installed in the hood or a container be available adjacent to the centrifuge for dunk decontamination of the rotor.

Users of these centrifuges feel that the potential hazards can be decreased by designing more dependable lip seals and face seals, by designing a biologically tight coolant system with provisions for adding fresh glycol and withdrawing contaminated coolant in a closed system, and by making the control console switches more versatile, such as the addition of switches to permit coolant flow without the need of having to turn on the vacuum for the post-run flushing to the coolant system.

Today, many laboratories are using the batch or continuous flow zonal type ultracentrifuge. Since each installation may be somewhat different, it may be necessary to determine initially, using a biological simulant, whether leakage occurs and whether there is aerosolization of fluid into the room. In addition, it is advisable to examine the equipment carefully to judge which parts are most likely to leak and take protective measures, if possible. For example, routine part changes and inspections should be done to minimize faulty components with the K-II continuous sample flow zonal centrifuge (26), namely: (a) change top lip seal after each run, (b) change bottom lip seal after three runs, c) inspect face seal after

each run, (d) change rotor end cap "O" rings after each run, and (e) check rotor spindle pivots after each run to assure a smooth running rotor. The catcher slinger drain container should contain iodophor equal to 2% when the container is full, and the coolant reservoir level is checked regularly to determine if seal leakage is occurring.

The following safety precautions should be observed when using large-scale zonal centrifuges:

- The centrifuge normally should be placed in a Biological Safety Cabinet under negative pressure when using hazardous agents. For high risk agents, the centrifuge and satellite apparatus should be contained in a Class III cabinet system. An alternative to the cabinet is a small negative pressure room with a leak tight door for maintenance access. The centrifuge in this small room could be operated remotely or through glove ports.
- The exhaust air from the containment room or safety cabinet should be filtered with HEPA filters.
- The exhaust air from the turbine drive should be passed through HEPA filters before being released to the atmosphere. Installation of a trap to remove oil mist from this exhaust air markedly extends the life expectancy of the filters. Vacuum pump exhaust air should be similarly filtered before release.
- Although some manufacturers recommend that the catcher-slinger fluid collections be purged through the turbine exhaust, there is an advantage in draining the collections to the exterior of the assembly. The discharge from the drain orifice, both air and fluid, must be contained; a disinfectant containing transparent receiver with a filtered vent is recommended. A similar trap and venting arrangement should be used on the rotor bypass lines for removing bubbles from the fluid feed lines.
- Process feed lines, especially those under positive pressure, should be kept as short as possible and have as few connections as possible. Utilization of 3- 4- and 5-way valves is suggested to facilitate the consolidation of the many lines in the system. A panel consisting of pump, flow meter, pressure gage, valves and stainless steel fluid lines, to which influent and effluent lines are attached, is suggested. For all external flexible lines, tygon tubing with a minimum of 1/16" wall thickness and polypropylene connectors having positive, strong fasteners are suggested. Additional safety is provided by nylon strap hose clamps on slip fittings. Operating pressure on feed lines should not exceed 15 psig. A pressure gage and flow meter should be mounted in the line between

the pump and the split to the top and bottom rotor feed lines. An added safety factor would be the provision of an audible alarm to the pressure gage. If possible, the feed line system should be pressure tested before introduction of biohazardous fluids. Testing is usually performed at the time the face seals are leak tested by filling the system with sterile buffer solution or water and the air is purged from the rotor.

- A peristaltic pump is recommended for energizing the flow of process fluids to the rotor. Although line pressure can be automatically regulated to a preset limit at the air pressure source, the pump method is recommended because it places only the line between pump and rotor under pressure. Because some pumps use soft rubber tubing, ballooning and rupture are possible; this tubing should be replaced frequently. It is recommended that the peristaltic pump, and as many of the lines as possible, be contained in a primary barrier.
- Gradient fractions should be collected within the confines of a primary barrier. A Class I or Class II cabinet is recommended. For high risk agents, a Class III cabinet should be used. An alternative is to perform the fraction collection within a plastic glove bag or box prefitted with the gradient discharge tubing running into it. After the fractions are collected, the entire bag and contents are removed to a safety cabinet for subsequent manipulation.
- The total system, including feed lines, effluent lines, coolant systems and ancillary equipment that may have contained or been exposed to hazardous agents, should be decontaminated prior to any breaking of lines or disassembly of the centrifuge. Gradient residue should be flushed from the rotor and core with warm water before the disinfectant is introduced. The rotor should be filled and flow reversed several times to assure contact of the disinfectant with all surfaces. Surface decontamination of the rotor exterior and shafts is suggested as it is raised from the rotor chamber. The end caps should be loosened and the rotor and core completely immersed in a disinfectant solution for initial cleaning. Special precautions should be taken against accumulations of debris in the shoulders of the end caps that the internal disinfectant may not have reached. All lines, influent bottles, reservoirs, connectors, etc., must be autoclaved before cleaning for reuse or discarding. For high risk agents, the decontamination with disinfectant should be followed by ethylene oxide exposure overnight at 60% relative humidity and 80°F for the entire cabinet and its contents. Ethylene oxide access to the rotor chamber and turbine air drive lines should be provided. After the centrifuge and external equipment (lines, pumps, valves, etc.) have been cleaned, reassembled, leak tested, and are ready for the next lot of material, they must be sterilized. For virus work, 70% ethanol is recommended; flow must be reversed several times and all bubbles removed to assure contact of the alcohol with all surfaces. The ethanol is drained and flushed out with sterile distilled water to minimize the possibility of residual concentrations that would

affect the virus. For bacterial and other organisms, particularly those that produce spores, alcohol may not be a suitable disinfectant. Regardless of what disinfectant is used, however, the system should be flushed out with sterile distilled water. In some instances, it may be necessary to flush the system with a sterile specific neutralizer for the disinfectant prior to the final flushing with sterile distilled water.

- Protective clothing, including a respirator, should be worn in areas where there is possible exposure to hazardous or infectious materials.
- No material should be removed from the special centrifuge room or Biological Safety Cabinet unless it has been decontaminated.
- The operator should be thoroughly familiar with this equipment to identify problem areas and to establish a system of replacing components likely to result in leakage.

6. Blenders, Mixers, Sonicators, and Cell Disruption Equipment

Hazardous aerosols are created by most laboratory operations concerned with blending, mixing, stirring, grinding or disrupting materials such as cells, tissues, blood samples, freeze-dried sera, and environmental samples that may contain infectious, toxic or otherwise hazardous materials. Even use of the mortar and pestle can be a hazardous operation. Ball mills, colloid mills, jet mills, tissue grinders, magnetic mixers, stirrers, sonic cleaning devices, ultrasonic cell disintegrators, and shakers are other devices that can produce hazardous aerosols.

a. Blenders and Mixers

The hazards associated with the liberation of aerosols during the operation of the blender have been recognized for many years. In one investigation (33), the air was sampled during the operation of two type of blenders; one was a one-liter plastic-capped bowl, the other was a 50 ml screw-capped bowl. The results, shown below, are indicative of the hazardous nature of aerosol release from a standard blender and also show how rapidly the aerosol decays within the blender.

Serratia indica recovered from aerosols produced during the operation of a blender

	<u>Serratia indica</u> recovered*	
	Plastic cap	Screw cap
During 2-minute operation	511	18
Cap removed immediately after blender was turned off	>2,100	>2,100
Cap removed 5 minutes after blender was turned off	306	629
Cap removed 90 minutes after blender was turned off	50	40

* Numbers refer to colonies appearing on sieve sampler plates. Sampling was a rate of 1 cu. ft. per min. for 2 min. during blending, and for 3 min following the operation. When the cap was removed, it was replaced after 10 sec.

Investigations were conducted on the particle size distribution of S. marcescens aerosols created during common laboratory procedures and simulated laboratory accidents (17). Over 1,600 viable particles per cubic foot of air sampled were recovered during blending operations and more than 93% of these particles were less than five microns in size. It was demonstrated that removal of the blender top immediately following the blending operation produced an aerosol with a mean concentration of 1,500 viable particles per cubic foot of air sampled.

The potential for accidental microbial aerosol transmission in the biological laboratory was discussed in 1973 at the Conference of Biohazards in Cancer Research held at the Asilomar Conference Center, Pacific Grove, California; it was reported' that blenders and homogenizers are particularly dangerous (34). It was recommended that these devices be used in a hood or other container that can be properly ventilated if the material is suspected of being pathogenic or allergenic. It was stressed that a gastight cabinet must be used in the event the material is highly pathogenic.

Highspeed blenders for safely processing infectious materials have been proposed. The unique features of one blender include (I) the elimination of gaskets and bearings at the bottom of the blender bowl by placing the bearings and drive motor at the top, outside the bowl, (ii) the provision for cooling the drive shaft and bearings with dry ice, (iii) the inclusion of a rubber washer in the lid, which is screwed onto the cup, and (iv) the fabrication of the bowl and lid from stainless steel. An air inlet and drainpipe are provided to allow for the removal of the contents without opening the lid, which would release an aerosol. This equipment is not commercially available; however, sufficient detail for fabrication has been published (35). Other studies (36, 37) concerned the infection hazards of the highspeed blender and presented means of correcting the problem by utilization of a new blender design. As an outgrowth of these studies, a safety container (Waring AS-1) for the Waring blender is commercially available that can be autoclaved or otherwise sterilized, has biologically inert Teflon bearings, a "standpipe"

agitator, a leak proof lid with an "O" ring gasket secured by swivel thumbscrews, and outlet fitting to allow the continuous flow of ingredients. The lid also has an outlet plug to permit easy removal of samples, as shown in the following figure.

[ILLUSTRATION OF CONTAINER (WARING AS-1) FOR THE SAFE BLENDING OF
HAZARDOUS MATERIALS.]

Magnetic mixers, although generally operated at slower speeds than blenders and not designed to create a turbulent, macerating action, are capable of creating aerosols, particularly if the material mixed produces bubbles or foam. Magnetic mixers provide a comparatively gentle, swirling action, but the mixing of infectious, oncogenic, allergenic, or toxic materials should be considered a potentially hazardous operation. Thus, the same safety concerns apply to magnetic mixers as to blenders.

b. Sonicators

Aerosol hazards associated with the operation of an ultra-sonic oscillator have been reported (38-39). Particles of infectious or hazardous materials can escape because of loosely fitting covers, loose gaskets at the bottom of the cup, or when the contents are removed from the cup. Use of a larger size "O" ring was found to provide a satisfactory closure. Modification of the cover to permit installation of a rubber diaphragm provided means for the safer removal of the contents by a syringe and needle. It also was recommended that sonicators be used in Biological Safety Cabinets.

Adequate decontamination of equipment potentially contaminated with infectious material prior to sonic cleaning is essential because of the hazard of creating aerosols during the sonic treatment (16). Wherever sonicators are used in a cleaning process, such as in dishwashers, animal cage washers, etc. , all items should be sterilized prior to cleaning.

c. Recommended Practices

Safe laboratory practices that are required generally when using blenders, mixers, ultrasonic disintegrators, colloid mills, jet mills, grinders, and mortars and pestles with hazardous biological or chemical materials are as follows:

- Operate blending and cell disruption and grinding equipment in a Biological Safety Cabinet.
- Use safety blenders designed to prevent leakage from the rotor bearing at the bottom of the bowl. In the absence of a leakproof rotor, inspect the rotor bearing at the bottom of the blender bowl for

leakage prior to operation. Test it in a preliminary run with sterile water, saline or methylene blue solution prior to use.

- If the blender is used with infectious material, use a towel moistened with disinfectant over the top of the blender. Sterilize the device and residual contents promptly after use.
- Glass blender bowls are undesirable for use with infectious material because of potential breakage. If used, they should be covered with a propylene jar to prevent dispersal of glass in the event the bowl breaks.
- A heat-sealed flexible disposable plastic film enclosure can be used for a grinder or blender. The safest practice is to use these within a Biological Safety Cabinet. That means they are not used for total containment but rather to spare gross contamination of the cabinet when equipment or procedures are used that are known to release aerosols.
- Blender bowls sometimes require supplemental cooling to prevent destruction of the bearings and to minimize thermal effects on the product.
- Before opening the safety blender bowl, permit the blender to rest for at least one minute to allow settling of the aerosol cloud.

7. Miscellaneous Precautions and Recommendations

Water baths and Warburg ,baths used to inactivate, incubate, or test infectious substances should contain a disinfectant. For cold water baths, 70% propylene glycol is recommended (22,25). Sodium azide should not be used as a bacteriostatic. It creates a serious explosion hazard.

Deepfreeze, liquid nitrogen, and dry ice chests and refrigerators should be checked and cleaned out periodically to remove any broken ampoules, tubes, etc., containing infectious material, and decontaminated. Use rubber gloves and respiratory protection during this cleaning. All infectious or toxic material stored in refrigerators or deep freezers should be properly labeled. Security measures should be commensurate with the hazards (22,25,40). The degree of hazard represented by contaminated liquid nitrogen reservoirs will be largely dependent upon the infectious potential of the stored microorganisms, their stability in liquid nitrogen, and their ability to survive in the airborne state. Investigations suggest that storing tissue culture cell lines in containers other than sealed glass ampoules might result in potential intercontamination among cell lines stored in a common liquid nitrogen repository.

It must be recognized that evacuating the atmosphere from a vacuum steam sterilizer prior to sterilization of contaminated material potentially can create a hazard by releasing infectious material to the atmosphere. This hazard can be prevented by installation of an efficient inline HEPA filter (water resistant; e.g., Flanders 7C81R-G) (41).

Ensure that all hazardous fluid cultures or viable powdered infectious materials in glass vessels are transported, incubated, and stored in easily handled, nonbreakable leakproof containers that are large enough to contain all the fluid or powder in case of leakage or breakage of the glass vessel (22-25).

All inoculated petri plates or other inoculated solid media should be transported and incubated in leakproof pans or leakproof containers (22,25).

Care must be exercised in the use of membrane filters to obtain

sterile filtrates of infectious materials. Because of the fragility of the membrane and other factors, such filtrates cannot be handled as noninfectious until culture or other tests have proved their sterility (22,25).

A variety of shaking machines are commercially available for aerating and mixing cultures, disrupting cells, culturing or homogenizing tissues, and mixing reactants in serological and biochemical studies. Since there is the possibility of glass breakage, closures becoming loose or dislodged, and the leakage of containers with the consequent release of aerosols or liquids to the environment, these machines should be used with caution and examined carefully for possible hazards associated with their use. Screw capped durable plastic or heavy walled glass containers should be used. If used for handling infectious or hazardous materials, the flasks, bottles, tubes, etc., should be held securely in place without undue strain on the container in leakproof trays. A plastic bag with or without absorbent material could be used to enclose the container as an additional safety precaution unless aeration requirements restrict enclosure of this type.

To prevent escape of infectious microorganisms during shaking, stoppers and cotton plugs of containers should be held in place with tape. Screw caps can be modified to allow diffusion of gases by drilling out the top and inserting an appropriate filter pad between the cap and the gasket.

Lacking a specially designed cabinet for shaking highly infectious materials, a completely enclosed shaker box fitted with an aerosol tight gasket or liquid seal can be fabricated. A glass wool filter in the lid or sides of the box permits diffusion of gases.

No person should work alone on an extremely hazardous operation (22,25).

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