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Working Safely with Biological Samples

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BIOSAFETY

Is There Such a Thing as a Nonpathogenic Organism?

The term "biohazard" is applied to any living agent that has the potential to cause infection and disease if introduced into a suitable host in an infectious dose. The "living agent" can include viruses, bacteria, fungi, protozoa, helminths (worms) and their eggs or larvae, and arthropods (insects, crustaceans) and their eggs or larvae.

We most commonly think of pathogenic microbes such as *Salmonella typhi* and *Leishmania donovani* as biohazards, but if introduced into a healthy body in large numbers (or an immuno-

compromised body in low numbers), organisms that are normally nonpathogenic can cause infection. The infectious dose will vary with the organism and the health of those infected. For example, *Shigella flexneri* requires the ingestion of only a few hundred organisms to cause intestinal disease. *Salmonella typhi* requires over a hundredfold more organisms to do so. Technically there is no such thing as a nonpathogenic microorganism.

Do You Know the Biohazard Safety Level of Your Research Materials?

Regardless of the type of work you will be doing with microorganisms, it is mandatory to know as much as possible about the safety precautions needed to handle the microbes you will be using, prior to entering the lab. Does your organism require special handling? Ask questions of the lab supervisor and your coworkers regarding the microbe itself, its safe handling and proper disposal. Know the location of first-aid kits, eyewash stations, and emergency lab showers.

In general, organisms used in the lab are classified in terms of the biosafety level (BSL) required to contain them, with BSL-1 being the lowest and BSL-4 being the highest levels, respectively. These classifications have been set by the U.S. government agencies such as the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, and its associated institution, the National Center for Infectious Diseases (NCID), the World Health Organization (WHO), and the governments of the European Community (EC). The CDC and NCID are an excellent source of information on the biosafety level classification of individual organisms, and the methods suggested for their safe handling. The CDC Web site is at *www.cdc.gov*, which has links to the NCID site. Another excellent source of information is a book entitled *Laboratory Acquired Infections* (C. A. Collins and D. A. Kennedy, 1999).

BSL-1 and BSL-2

BSL-1 agents present no, or minimal, hazard under ordinary conditions of safe handling. The common host cells used in cloning experiments are classified as BSL-1, such as *E. coli* and *Saccharomyces cerevisiae*, and they can be handled at the benchtop. Simple disinfection of the workbench after handling and good hand-washing will be sufficient to eliminate organisms from any spillage. Low-level pathogens such as the fungus *Candida albicans* or the bacterium *Staphylococcus aureus* can also be safely handled at the benchtop, if the organism does not come into contact with

the skin, or mucous membranes. Care should also be taken when handling sharps that may be contaminated with a microbe. A contaminated scalpel blade, needle, or broken glass can serve as a vehicle of entry to the body. As described more thoroughly below, wearing a lab coat, safety glasses, and disposable gloves is always a good idea when handling any quantity of microorganism. The handling of liquid culture could also lead to aerosolization of the microbial suspension, hence wearing a particle mask might be useful as a precaution. It is always best to take an approach that maximizes your own safety and the safety of those around you.

BSL-2 agents possess the potential for biohazard, and they may produce disease of varying degrees of severity as a result of accidental laboratory infections. Moderate level pathogens, such as *Neisseria gonorrhoeae*, are classified as BSL-2. A safe way to handle BSL-2 organisms is in a laminar airflow, biosafety hood. By generating a flow of air inside the cabinet, these hoods are designed to keep aerosols from leaving the hood and entering into the room's airspace. The hood's proper function should be certified yearly by professionals. The Environmental Health and Safety officers of your institution or outside contractors can perform this function.

Usually a germicidal UV light is used to disinfect the inside of the hood when not in use. As a precaution prior to use of the hood, the airflow should be on for 15 minutes while the germicidal light is on. A wipe-down of the hood's inside working surface with 70% ethanol is also a useful precaution after the UV light is turned off. The airflow should be kept on during the entire time the hood is in use, and the glass panel on the front of the hood should be raised only high enough to allow comfortable use of the worker's arms inside the hood. Most hoods are equipped with an alarm to warn the worker if the front panel is raised too high.

Basic microbiological techniques of sterility, a minimum of protective gear, disinfectant, and common sense are all that are required to safely handle BSL-1 microbes used as cloning or expression vectors in the laboratory. Under common sense and in accordance with safety regulations, there should be absolutely *no* eating or drinking by an individual during the handling of a microorganism in the lab. Those workers in a diagnostic microbiology lab or doing research on a BSL-2 pathogen will wish to use a biosafety hood when necessary, along with protective clothing.

BSL-3 and BSL-4

Any organism requiring BSL-3 or BSL-4 containment should only be handled by highly trained individuals, using extensive safety precautions. Training in a lab that has experience with the microbe is highly recommended.

BSL-3 pathogens pose special hazards to laboratory workers. They must be handled, at very least, in a biosafety hood. No open containers or those with the potential to break easily should leave the hood if they contain the BSL-3 agent. Eye protection, particle mask, lab coat, and gloves are mandatory. Centrifugation of such organisms requires sealed containers to prevent aerosol and spillage.

Virulent BSL-3 pathogens that cause disease in low numbers and are transmitted by aerosol, such as *Mycobacterium tuberculosis*, require environmentally sealed containment rooms and also require the worker to be completely protected by special clothing, colloquially referred to as a "moonsuit," because it resembles the type worn by astronauts. Disinfection and removal of the suit is required before the wearer can enter the open environment.

BSL-4 pathogens pose an extremely serious hazard to the laboratory worker. These are the "hottest" pathogens, such as Ebola virus. Only a few places in the United States and elsewhere in the world are equipped for such studies. Only highly trained professionals are qualified to handle these agents.

How Can You Learn More about the Genealogy of Your Host Cells?

If the cell is obtained from a commercial source, such as a biotechnology company or the American Type Culture Collection, then the background on the host cell is often provided with the cell stock or in the catalog of the company. For strains of *E. coli* commonly used for cloning, the catalogs of biotechnology companies often have appendixes that list phenotypes and original references for the given strains. If the cell comes from a personal contact (i.e., another scientist), then be sure to ask for references or technical material on the cultivation and use of the cell. If possible, try to reproduce the desired cellular activity in a small pilot experiment prior to using precious materials or resources in a large-scale study. A wealth of genetic information and links to *E. coli* resources is available at *http://cgsc.biology.yale.edu/*.

Are You Properly Dressed and Equipped for Lab Work?

Regardless of the level of one's experience in the laboratory, it is wise to be prepared for the worst in terms of accidents. Proper preparation starts with proper protection for yourself and your co-workers. A number of common accessories should be used as needed.

Lab Coat

Most laboratories require the wearing of a lab coat, but even when not mandatory, a lab coat is a good idea. Protection of clothing and, more important, the skin underneath, is worth the effort and (perceived) inconvenience. For those working with microbes, clothing can be permanently fouled by a spill of almost any microorganism. Even a small break in the skin can serve as a portal of entry for a seemingly innocuous microbe that can result in a serious infection if the microbes gain access to the circulation in sufficient numbers. For those working with flames, such as a Bunsen burner, some institutions require a lab coat of flame retardant material. This ensures an added level of protection should there be a spill of a flammable liquid followed by ignition at the lab bench.

Closed Footwear

The open-toed shoe, sandal, or "flip-flop," even while wearing socks, provides easy access to the foot for sharps, hazardous chemicals, and infectious agents. The protection afforded by a closed-toe shoe against these assaults could provide an important level of safety. When choosing between fashion, or even comfort, and protection, protect your feet!

Eye Protection

It is strongly suggested to wear some form of eye protection when handling large volumes of microorganisms to protect against splashing during handling. Even a nonpathogen can set up infection when introduced in large numbers on the conjunctiva. Eyeglass (but *not* contact lens) wearers are afforded reasonable protection in these circumstances, but more safety is provided with the larger protective surface afforded by safety goggles or glasses, which can fit over conventional eyeglasses if necessary. For individuals working with pathogenic organisms, it is essential to wear eye protection at all times.

Conventional safety glasses are suitable against BSL-1 and BSL-2 class pathogens. For BSL-3 pathogens, a full-length pro-

tective face mask will provide additional defense against accidental exposure of the face and eyes.

Latex Gloves

The wearing of protective gloves is a good idea if the skin on the hands is abraded or raw. It is very important to wear protective gloves if a pathogen is being handled. Some people's hands can be irritated by the powder on many types of latex gloves. Most can be obtained in powder-free form. Less fortunate individuals are allergic to the latex in the gloves. Cloth glove inserts are available that can prevent contact of the latex with skin.

Heavy-Duty Protective Gloves

For those individuals handling pathogens and sharps simultaneously, such as during inoculation of an experimental animal, it is a good idea to wear a heavier rubber glove over the latex glove. Removal of the last 0.5 to 1 inch of the finger tips of the heavy glove will afford the worker with the dexterity to handle instruments or other items with efficiency, and still provide protection to the bulk of the hand.

Safety Equipment and Supplies

The two most important pieces of safety equipment in any lab are the eyewash station and the emergency lab shower. These two equipment stations should be regularly tested (every 6–12 months) to be sure they are fully operative. Know their location. In the event of the splashing of a microbial suspension in the eye, if possible, go directly to the eyewash station and flush the eye thoroughly with water. Then seek medical attention immediately. Even small numbers of microbes can permanently damage the eye if it is left untreated. The lab shower is very useful in the case of a chemical spill, and a large spill of a serious pathogen on the body surface also could be removed in part by rinsing under the lab shower. Again, seek medical attention following such a circumstance. Finally, every lab should have a well-stocked first-aid kit for treatment of minor mishaps, and to provide intermediate care for more serious accidents. Take it upon yourself, or appoint a lab safety officer, to be sure the first-aid kit is stocked and the wash stations are operative.

Are You Aware of the Potential Hazards during the Setup, Execution, and Cleanup of the Planned Experiment?

In the microbiology lab there are physical, chemical, and microbial hazards. When handling a hazardous material or performing

a hazardous procedure, the most important thing is to pay attention to what you are doing and, if transporting the material, where you are going. Wear the protective clothing as outlined in the previous section. Do not stop to answer the phone or chat. No clowning around; this is not a time for levity. Even an act as simple as sterilizing an inoculating loop can be hazardous if you get distracted.

The physical hazards include burns and cuts from sharps. Burns can result from a Bunsen burner or gas jet, an inoculating loop, a hot plate, or the autoclave. When handling items going in or coming out of the autoclave, heavy-duty cloth gloves designed for handling hot containers are essential. Materials heated on a hot plate or in a boiling water bath should also be handled with heavy duty protective gloves. Burns can also result from ignition of flammables like ethanol or acetone. *Always* keep containers with these liquids safely away from a heat source. Malfunctioning machinery can also be a source of a burn or a fire. A pump motor that has seized can cause a fire. If you smell smoke or other toxic gases emanating from a piece of equipment try to turn it off or unplug it immediately, and call the fire department if necessary. If this action seems unsafe, call the fire department immediately.

Needles, broken glass, scalpels, and razor blades are all potential hazards. Pay attention when handling them and dispose of them properly. Most all labs require disposal using a certified "sharps" container, and removal by housekeeping staff or health safety workers. *Never* throw a sharp into the everyday trash. This is a potential hazard and possible source of infection for the housekeepers.

Nearly all microbiology labs utilize corrosive acids, alkalis, and organic compounds that are toxic. The potential for toxicity can manifest itself through amounts as small as the fumes released by opening the container. Even a whiff of a concentrated acid or other corrosive liquid can cause tissue damage to the nasopharynx. A spill of even a few hundred milliliters of an organic chemical, like phenol, on the body can be life-threatening. Phenol vapors, in excessive amounts, can cause damage to the nasopharyngeal mucosa and to the mucous membranes of the eye. Brief exposure to phenol vapors can cause minor irritation of these mucosa as well. If one uses phenol frequently, it is sensible to perform the manipulations in a chemical fume hood if possible.

Sources of microbial contamination to you and others are aerosols formed by the handling of the inoculating loop, prepara-

tion of slides, plating of cultures, the pouring of microbial suspensions, and pipetting. These procedures can be serious sources of infection if a hazardous pathogen is being handled. Each of these actions will be discussed individually. For individuals whose body defenses are compromised by underlying disease or medical treatment, it is sensible for them to check with their physician as to the potential hazards to them of working in a microbiology lab where even organisms that are normally nonpathogenic are being handled.

The Inoculating Loop

Excessively long or improperly made loops can shed their inoculum, either by vibration or spontaneously. A film formed by a loopful of broth culture that is vibrated can break the surface tension that keeps the film in place, forming an aerosol. The longer the loop is, the more vibration that ensues from handling. An incompletely closed loop can also easily result in a break in surface tension of the film. The optimal size of loop is approximately 2 to 3 mm in diameter, and the loop should be completely closed. The length of the wire portion of the loop, or shank, should be approximately 5 to 6 cm. If a large flask is being inoculated, tilting the flask to bring the liquid closer to the neck may be a way to avoid the use of a very long wire. When loops become excessively bent or encrusted with carbonized material, they should be replaced. Pre-sterilized, single-use plastic loops are also available. They are not to be placed in contact with flame or solvents such as acetone, and should be discarded into a disinfectant solution.

The discharge of proteinaceous or liquid material that often follows flaming a loop has been suspected as a source of contamination, but there is little evidence to support this contention. Nonetheless, it is best to decontaminate the wire loop by placing it into the apex of the internal blue flame of the burner so that any discharged material has to pass through the bulk of the flame as it leaves the loop.

Preparation of Slides

The production of aerosols by spreading of a bacterial suspension on a slide is minimized by gentle movements, especially when removing the loop from the spread suspension. For pathogens this activity is best performed in a biosafety hood, and the slides should not be removed from the hood until completely dried.

Streaking of Plates

In general, aerosol production is minimized by using smooth plates. Rough surfaces or bubbles in the agar can lead to excessive vibration of the loop. Spreading of samples with a sterile glass rod may minimize the production of aerosols on agar plates with rough surfaces.

Pouring of Microbial Suspensions

Following centrifugation, pouring off the supernatant from a microbial suspension can result in aerosols. One safe way to minimize aerosol production is to use a funnel with the narrow end submerged into disinfectant in a large container. Use enough disinfectant to mediate the decontamination of the supernatant, and a container large enough to avoid overflow. A volume of disinfectant equal to, or greater than, the amount of supernatant to be decontaminated should suffice. Rinse the funnel with more disinfectant to handle any residual supernatant clinging to the funnel wall.

Pipetting

Even for solutions deemed to be safe, it is good practice to *never* mouth pipette. It is mandatory not to mouth-pipette a microbial solution, even with a cotton-plugged pipette. Aspiration of organisms into the mouth can occur despite the cotton plug. Blowing out the last few droplets from a pipette can also form aerosols. Use either a manual or automatic pipetting aid to pipette. The discharge of the last few droplets using either manual or automatic pipette aids can result in aerosols, so avoid this if possible. If it is necessary to discharge the entire contents of the pipette, try to avoid spraying. Again, for serious pathogens, pipetting should be performed in a biosafety hood. Contaminated pipettes should be discarded into a container containing a sufficient volume of disinfectant to permit the *complete* immersion of the pipette.

Are You Prepared to Deal with an Emergency?

Adequate preparation for an emergency requires both an appreciation for the potential hazards involved and knowledge of the resources available to handle the emergency. Such preparation should precede actual lab work, although this is rarely done. We have discussed the appropriate types of laboratory clothing that should be worn in the microbiology laboratory, important safety equipment and supplies, and potential sources of harm.

There are several potential sources of an emergency situation in the lab.

Flammable Liquid and Microbial Culture Spills

In case of a flammable liquid (i.e., ethanol) spill, turn off any sources of ignition immediately, such as a Bunsen burner or hot plate. Then try to contain the spill. If the ethanol spilled onto your clothing, be careful not to ignite the clothing while trying to turn off potential sources of ignition. Remove the wet clothing as soon as possible.

For the microbial spill, contain the spill with disposable cloth or paper and decontaminate the area with a disinfectant. If any microbial suspension has contaminated your clothing, remove and disinfect the clothing. Any spill onto the skin should be washed off as soon as possible. If necessary, use the eyewash station or emergency shower.

Accidental Inoculation of Self with Microbe; Nonpathogen versus Pathogen

Accidental inoculation with any microbe should be treated as a serious situation, regardless of whether the organism is considered pathogenic or not. Even "cloning strains" of *E. coli* can cause septicemia if inoculated in substantial numbers. Seek immediate medical attention. Accidental inoculation of oneself with a syringe containing blood or other human tissue fluid should also be considered a medical emergency. Animal blood or tissues can also contain serious pathogens. Do not let accidental inoculation pass unattended; go immediately to seek attention from a medical professional. Be prepared to provide as much information as possible to the health care provider regarding the microbial agent or source of biological fluid.

Fire and Burn to Self or Fire in Surroundings

A direct burn from a Bunsen burner flame or hot plate can be serious. Even a minor burn can become infected. Seek medical attention if necessary. If one's clothing catches fire, try to remove it immediately or roll around on the floor to extinguish the flame. This is no time for modesty! Burning clothing can result in a life-threatening or fatal burn. Fire in one's surroundings can also develop into a life-threatening situation. If the fire is of a small, manageable scale, and you know the proper technique for extinguishing the different types of fires, you might attempt to stop the fire yourself. However, many fires are not easy to extinguish, and

can be spread by incorrect use of extinguishing equipment. If you have any doubts, immediately leave the area and pull the fire alarm or call the fire department.

Electrical Hazard from Malfunctioning Equipment

Be aware of frayed electrical cords, which should be repaired or replaced immediately. Any piece of electrical equipment that produces sparks or smoke has the potential to cause harm by electric shock or fire. If in doubt, leave the room and call the fire department.

What Are the Potential Sources of Contamination of Your Experiment and How Do You Guard against Them?

Two prominent sources of "contamination" are (1) the introduction of microbes from the environment and (2) a mix-up of two or more closely related strains of organisms that you are working with simultaneously.

Environmental contaminants are most commonly fungi from the air that grow rapidly as fuzzy colonies on a bacterial plate. They are not harmful to the healthy researcher but can wreak havoc on your bacterial or mammalian cell cultures. Once a fungal contamination of this type occurs, it is nearly impossible to get rid of it. The best course of action is to get a fresh aliquot of the lab strain you're working with and start over again. Once the contaminant is visible by the naked eye, it has already produced numerous spores that will be spread as soon as the Petri dish lid is opened, making it difficult to go into that plate in order to pass the bacterial strain onto a fresh plate. You will only carry the spores from the contaminant with you. This speaks to the necessity of backup cultures of important organism strains, whether they are viruses, bacteria, fungi, protozoa, or higher eukaryotic cells. If the only copy of an important strain is contaminated by airborne fungi, heroic measures including treatment of the culture with antifungals such as amphotericin B or nystatin can be attempted, but this approach is usually not successful since the entire fungal population is not killed. Seal the contaminated plate with either tape or paraffin film, and discard it in the biological waste for autoclaving. For contaminated cell culture flasks, keep them sealed and dispose of them by autoclaving.

To prevent contamination from environmental sources, adhere to good sterile technique. Flame the inoculating loop thoroughly, leave the lid of the Petri dish or medium bottle open for as short a period of time as possible, and avoid working in a drafty area.

If environmental contamination persists, work in a laminar flow biosafety hood. If a contamination problem persists in the lab, consider an examination of the lab's air delivery system. Placing filters on the air ducts may reduce levels of contamination. Also consider lab clothing or dirty hands as a possible source of contamination. If circumstances dictate that microbial cultures and mammalian cell cultures be handled in the same hood, the working surfaces of the hood should be decontaminated with disinfectant after working with bacteria, and the germicidal lamp should be on at least 30 to 60 minutes before using the hood for cell culture.

The second possible source of contamination is by introducing a closely related strain to your culture while working with both strains simultaneously or consecutively. Such a mix-up is quite possible unless you are very attentive. It is difficult, if not impossible, to distinguish different *E. coli* strains by the naked eye at the level of colony morphology or microscopically by Gram staining. It may only be possible to do so on the basis of genotypic or phenotypic markers. Try to avoid handling more than one strain at a time. Most important, label all plates, tubes, and flasks thoroughly. If multiple strains are to be handled simultaneously, using different colors for the labeling of different strains is helpful. If a mix-up is suspected, put the strain of interest through as many types of tests as necessary to confirm its identity. This will save time and money in the long run compared to continuing to work up a contaminant, in which case you will need to start over anyway.

How Should You Maintain Microbial Strains in the Short and Long Terms?

For the short term, most bacterial strains can be maintained on plates without subculturing at 4°C for two weeks. Store plates inverted (with the half containing the agar facing you), because condensation on the plate lid of water evaporating from the agar can drip down onto the plate, and cross-contaminate isolated colonies as the liquid spreads. It is possible to remove excess liquid from the lid by a flick of the wrist into a lab sink and then using a disposable tissue to blot the excess liquid. This may introduce a contaminant, but it's a trade-off between that and soaking the plate with the condensation.

In the clinical microbiology lab, blood agar plates that contain cultures under evaluation are stored at the benchtop for up to 7 to 10 days after the initial overnight incubation at 37°C.

The concern here is desiccation of the agar over time and subsequent death of the microbe. Wrapping the plates in plastic wrap or sealing the edge with Parafilm® will help prevent desiccation.

For the long term, most microbial strains can be stored in glycerol (10–15% final concentration) at either –20°C (1–2 years) or –80°C (>2 years). There is no need to thaw the entire culture to recover the strain. A scraping of the frozen stock with a sterile toothpick or inoculating loop and inoculation of a plate is sufficient to recover the culture. Repeated freeze–thaw cycles will decrease the longevity of the frozen culture.

How Do You Know If Your Culture Medium Is Usable?

If liquid microbiological media remains sterile, it is usable for long periods of time (years) for most strains. Some fastidious microbes, such as the streptococci, are more sensitive and require freshly prepared media due to the lability of critical nutrients. In a clinical microbiology lab, where quality control is important, media should not be used past the expiration date for diagnostic purposes, but it may be used for less critical tasks.

Any medium that shows signs of contamination should not be used and should be discarded appropriately. Do not try to re-sterilize the medium and use it, since the contaminating microbe has not only depleted nutrients from the medium but has released or shed products that could interfere with the growth of your strain. It is easier and cheaper to simply make fresh medium.

Are Your Media and Culture Conditions Suitable for Your Experiment? How Significant Is the Genotype of Your Microbial Strains?

These two questions are related since the genotype of the organism will influence medium composition and growth conditions. Knowing the genotype of the organism in relationship to auxotrophic markers is critical, since essential nutrients, such as an amino acid or nucleoside, may need to be added to the basal medium to permit growth of the strain. Knowing the genotype of the organism in relationship to antibiotic resistance markers is critical to allow selection of the correct strain in the presence of organisms sensitive to the specific antibiotic.

What Are the Necessary Precautions and Differences in Handling of Viruses, Bacteria, Fungi, and Protozoa?

Since each of these four main groups of microbes have members that are either overt or opportunistic pathogens, a preliminary

understanding of the pathogenic potential of the specific organism you will be working with is warranted. Part of this understanding is that of your own susceptibility to infection by the organism with which you are working. If you are in good health, then those organisms classified as opportunists should pose no threat unless accidental inoculation occurs. Individuals who are immunocompromised are at risk from infection from organisms that cause no harm to the healthy person. For overt pathogens, the appropriate precautions of laboratory apparel and, if necessary, a biosafety cabinet, applies to all workers.

Viruses of bacteria, bacteriophages, are not believed to pose a threat to humans and can be handled at the benchtop. Accidental ingestion of phage could, potentially, perturb the normal bacterial flora of the gut and possibly lead to diarrheal disease. As always, avoid mouth-pipetting to eliminate this possibility. Animal viruses, and those that infect plants as well, are propagated inside their respective eukaryotic cells. Again, plant viruses pose no health threat to humans. Many animal viruses that are conventionally handled using BSL-1 and BSL-2 containment can pose a threat to one's health, however, and should be handled carefully. This includes using the appropriate lab safety gear, taking care while handling sharps or glassware that have come in contact with the virus, and avoiding pipetting by mouth. Cell culture is highly susceptible to contamination from organisms in air and water droplets, hence is routinely performed in a biosafety cabinet with laminar airflow. This protects the cell culture from environmental contamination and has the benefit of protecting the worker from exposure to the virus. There is always the potential for accidental inoculation via a sharp object, or by a spill that occurs while handling viral stocks or infected cells.

Many of the precautions for handling bacteria have been described in the earlier discussion of biosafety. Inoculation via sharps, aerosols, and spills are the most common means of infection during handling.

Spore-forming filamentous fungi, like *Aspergillus*, pose the risk of infecting the lab worker by the release of the spores that are an integral part of their life cycle. They are easily made airborne and inhaled. They should be handled in a biosafety cabinet with laminar airflow. Inoculating loops and needles for passing fungal strains are sterilized and decontaminated with special electric heating coils shielded with a metal or ceramic covering to protect the worker from accidental burn. This mode of sterilization replaces the flame burner, which can be difficult to manage in a biosafety cabinet with laminar airflow. Non-spore formers

such as *Saccharomyces cerevisiae* and *Candida albicans* can be handled at the benchtop using sterile technique. More serious pathogens in the non-spore former category that cause respiratory disease, such as *Coccidioides immitis* and *Histoplasma capsulatum*, should always be handled in a biosafety cabinet with laminar airflow.

Nonpathogenic protozoa that can be grown in axenic (pure culture; no feeder or accessory cells) culture can be handled at the benchtop using sterile technique. All pathogenic protozoa, grown in either axenic culture or in cell culture, should be handled in a biosafety cabinet with laminar airflow. They can become airborne in aerosols and make contact with the soft tissues of the eye, nose, oral cavity, and throat. Pathogenic hemoflagellates, such as those of the genera *Leishmania* and *Trypanosoma*, can penetrate the mucosal epithelium and establish infection.

While many pathogens pose the threat of infection, proper lab gear, safety equipment, and training permit their safe handling. Know the potential hazards before beginning work. Be sure the necessary supplies and equipment are available and accessible to deal with an emergency, should one arise. Above all, don't panic, and seek help immediately should an accident occur.

What Precautions Should Be Taken with Experimental Animals?

The Infection of Experimental Animals by a Natural Route or by Inoculation

Several techniques are used to infect experimental animals, depending on the nature of the infection to be induced. Each procedure has its attendant hazards for the investigator. Aerosols, contact of skin or mucosal surfaces by contaminated clothing and gloves, and inoculation by sharps are common hazards.

Respiratory infections are commonly initiated by (1) natural acquisition of the infection by co-housing the recipient animal with an infected donor animal, (2) direct introduction of the infectious agent via intubation of the lung, and (3) exposure of the animal to the infectious agent via aerosol in a sealed chamber. Aerosolization via mode (3) is performed with serious pathogens such as *Mycobacterium tuberculosis* and requires special containment rooms as well as airtight suits on the part of the investigators.

Mucosal pathogens that infect the nasopharynx, oral cavity, eye, gastrointestinal tract, or genitourinary tract are applied by direct inoculation, using a swab containing the infectious agent in buffer or medium. Gastrointestinal pathogens can also be applied by

feeding the infectious agent to the animal or by gavage (direct intubation and inoculation). Accidental inoculation of the mucous membranes by the contaminated glove or sleeve of the worker is a common mode of transmission.

In particular, the inoculation of an animal by injection with a needle is particularly hazardous if the animal is not anesthetized or restrained during the procedure. Wriggling, other rapid movement of the animal, or an unrelated distraction during inoculation can readily result in accidental inoculation of the handler. If the animal cannot be anesthetized or restrained, assistance by another person in either the holding or inoculation of the animal can help minimize accidental inoculation of the investigator. Avoid unnecessary distractions and pay attention to what you are doing.

Safe Handling of Infected Experimental Animals

A reasonable precaution for the safe handling of infected experimental animals is to learn the techniques for safe handling of the same animal species when it is uninfected. This always involves minimizing the aggravation of the animal. First and foremost, be calm. Like pets, lab animals can sense nervousness and fear. Be firm but gentle. Hold the animal in a way that is not painful or hazardous to the animal. For example, improper handling of rabbits can lead them to contort themselves in a way that they damage their own spine, which results in having to sacrifice the rabbit. Trained laboratory animal medicine personnel are the best source of information and technique for the novice. In the infected animal, the hazards are increased by the fact that the animal may feel poorly or be irritable as a result of the infection. Those animals that have been injected will remember that experience and may be reluctant to be handled again. In some cases a bite or a scratch by the animal could serve as a means to transmit the primary infection, or another microorganism, to the handler.

Disposal of Carcasses, Tissues, and Body Fluids of Infected Experimental Animals

First and foremost, *never* place these materials in the regular trash. Wrap carcasses and tissues in a plastic bag and seal the bag. Do not autoclave carcasses or large amounts of tissue. For temporary storage, freezing the tissue or carcasses in the plastic bag at –20°C is acceptable until proper disposal can be performed. Most institutions that undertake animal work have

specific facilities in their vivarium for disposal of tissues by incineration. Check with the vivarium personnel for the proper protocols. Fluids such as blood from an infected animal, and very small amounts (a few grams) of tissue can usually be autoclaved with the other infectious waste, and disposed of properly.

What Precautions Should Be Considered before and during the Handling of Human Tissues and Body Fluids?

Any fresh or fresh-frozen human tissues or body fluids should be considered to be potentially infectious, and handled with the utmost care. When handling human tissues, there are always risks of exposure to hepatitis, HIV, and *Mycobacterium tuberculosis*, to mention only a few pathogens. Proper lab attire and use of a biosafety cabinet with laminar airflow, is essential. Further containment in a BSL-3 facility may be warranted under the appropriate circumstances. Get as much information as possible as to the origin of the material and potential risk factors before handling the samples.

Any materials or equipment that comes into contact with the tissues should be decontaminated by treatment with disinfectant. Any extraneous tissue fragments or fluids left over from the work should be sterilized by autoclaving. Disposal of large pieces of tissues should be handled under the supervision of the institution's Department of Environmental Health and Safety, which will likely incinerate the material much like is done with animal carcasses. They will also be able to answer general questions about risk factors, and other disposal procedures. In the absence of institutional procedures, use common sense and approach the situation as if the biohazard risk is high.

If the tissue is properly and thoroughly fixed, then the risk of infection is eliminated and the material can be handled at the benchtop. Avoid accidental ingestion of the material via eating and drinking in the lab. The preparation of fixed tissues and safety considerations for the handling of contaminated tissue specimens is comprehensively described in the text by Prophet et al. (1992).

What Is the Best Way to Decontaminate Your Work Area after Taking down Your Experiment?

No matter how harmless the microbial agent that you have worked with may be, it is always a good idea to decontaminate your work area when finished. While many microbes do not survive on dry surfaces for prolonged periods, others such as

Staphylococcus aureus, do. If you have used absorbent bench paper to cover the lab bench, dispose of it in the biohazardous waste.

If you have worked directly on the benchtop, there are numerous disinfectants suitable for use on these surfaces. A wide variety of additional disinfectants are commercially available. They are inexpensive; use them liberally. Not only does this reduce the risk of transmitting infection to yourself and others, but it also reduces the risk of contaminating your next experiment with organisms left at the benchtop.

There are several groups of disinfectants, each with their specific uses and advantages (Jensen, Wright, and Robinson, 1997).

- *Alchohols.* Wiping the bench with a solution of 70% ethanol is commonly done. Ethanol and isopropanol both kill microbes by disrupting cytoplasmic membranes and denaturing proteins. They kill most vegetative bacteria (but not endospores), viruses, and fungi. Ethanol and isopropanol concentrations of 70% to 90% are more microbicidal than concentrations above 90%, since they require some hydration for antimicrobial activity. Ethanol is also very effective as a skin antiseptic.
- *Heavy metals.* Various mercury-containing compounds were used as disinfectants in the past, but are rarely used now because of their toxicity. Dilute (1%) silver and copper ion-containing solutions are also used as disinfectants but are commonly used in water purification. They act by combining with proteins, such as enzymes and inactivating them.
- *Phenol and its derivatives.* These act by damaging cell walls and membranes and precipitating proteins. These are toxic in concentrated form, but are often mixed at low concentration (3%) with detergents or soaps to provide added disinfectant capacity on skin. Their advantage is that they are not inactivated by organic matter. Dilute solutions of phenols are found in many of the commercial disinfectants used on lab benchtops. Lysol™ spray, a very effective surface disinfectant, is a mixture of 79% ethanol and 2% *o*-phenylphenol.
- *The halogens.* The halogens iodine and chlorine are useful chemical disinfectants that oxidize cell proteins. Hypochlorites (0.5%) are the active agents of household bleach. The halogens are inactivated by organic matter and can lose their effectiveness when excessive organic matter is present in solutions and on surfaces. Iodine (2%) is an effective skin disinfectant, and is a component of some disinfectant soaps.

- *Quaternary ammonium compounds.* These compounds have detergent activity and solubilize cell membranes. They are commonly used at concentrations of 1% or less to disinfect floors, benchtops, and other inanimate objects. They are inexpensive, odorless, and nontoxic, but they are not as microbicidal as the aforementioned compounds.

Is It Necessary to Decontaminate Yourself or Your Clothing? Is There Significant Risk of Contaminating Others?

The answer to both of the above questions is a resounding *yes*! This is particularly important in a hospital setting where you will likely encounter patients that may be highly susceptible to infection. Recent studies (Neely and Maley, 2000) have shown that gram-positive bacteria, particularly multidrug-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus, can survive for prolonged time periods on common hospital and laboratory fabrics. Of 22 different organisms tested, all survived at least a day and some for more than 90 days! This study alone provides a good reason not to wear your lab coat to the cafeteria. Leave your lab wear, including lab coats, in the lab. Get your coat laundered with regularity. Any article of clothing, whether lab attire or personal clothing, should be washed very soon after an accidental spill of a microbial suspension or contaminated material onto it. Even if you have been wearing latex gloves, wash your hands when finished working. These simple control procedures will go a long way to stop the spread of infection to yourself and to those around you.

MEDIA PREPARATION AND STERILIZATION

How Can You Work Most Efficiently with Your Media Preparation Group?

Learn the Capabilities and the Guidelines

Media preparation facilities serve a crucial role for large numbers of researchers, and they are usually extremely busy. The more familiar you become with the operational guidelines and functional capabilities of the facility, the more likely you will get the materials you need when you need them.

Get to Know the Staff

Learn the media group's supervisory structure, and let the staff get to know you. As with any situation where a facility has to serve the needs of many different people, getting along with those that

do the work will benefit you in the long run. A courteous, considerate, and respectful approach is always rewarded by an extra effort on the part of the staff to get your work done in a timely manner. Media preparation is physically demanding and requires more technical skill than you might initially imagine. "Thank you, I appreciate your help" goes a very long way. When things go wrong, seek out the responsible individuals and make your concerns known in a civil manner.

Show Consideration for Their Safety

Notify the personnel of any potential hazard in the job you want them to do. If there is broken glass or other sharps in material to be decontaminated, advise them of the hazard. If the material they will be handling contains an agent that is considered a biohazard, inform them and discuss ways that the material can be handled safely. It is your responsibility to learn which chemical or biological materials require special disposal. Organic reagents such as phenol, or animal parts and bedding, are examples of materials that should not be sent to the typical media preparation facility.

Radioactive material should never be included with material destined for decontamination by media room personnel. The disposal of this material should be handled by authorized personnel from your institute's Radiation Safety or Health Physics office.

Which Autoclave Settings Are Appropriate for Your Situation?

Liquids

For liquids, use the liquid cycle with slow exhaust (exhaust rate describes the speed with which steam exits the sterilizing chamber). Liquids need a slow exhaust to ensure that the steam in the chamber does not depressurize so fast as to cause the liquids to boil over or evaporate excessively, as occurs with a fast exhaust setting. However, if the liquids and other materials are to be discarded, a fast exhaust rate can be applied.

Nonliquids

For nonliquid materials, use the wrapped, or gravity, cycle with fast exhaust. For sterilization of dry items (pipettes, instruments, test tubes, etc.) you would use the wrapped or gravity cycle. The fast exhaust serves to remove most of the excess moisture that accumulates during steam autoclaving. Sometimes a period of drying in a warm room (usually overnight) is required to

evaporate any excess moisture that remains after the fast exhaust cycle.

Some of the newer autoclaves don't have a cycle that provides only fast exhaust; here you would have to use either the liquid cycle or the gravity cycle, which would have the dry cycle built in.

What Is the Best Wrapping for Autoclaving? Aluminum Foil, Paper, or Cloth?

For dry materials such as surgical instruments, the best wrapping is cloth; paper is the second choice. However, as long as the aluminum foil is free of holes, it is the easiest and fastest method of wrapping. If wrapped properly, items in cloth and paper will stay sterile as long or longer than items in foil. They can be re-sterilized without repackaging. Although foil can be used several times to cut down on waste, repeated autoclaving can break it down. Reused foil should be checked for pinpoint holes or cracks by holding it to the light. Pipettes and surgical instruments can also be placed in metal canisters, metal pans with lids, or glass tubes with a metal cap for sterilization. Paper sterilizing bags are available, including those with see-through plastic on one side, so you can see the contents of the bag.

For liquids, use a bottle with a screwcap or a flask with a covering. In bottles, leave approximately 25% to 30% of the bottle volume empty to allow for liquid expansion during autoclaving. *Never* autoclave a tightly sealed bottle; it could crack or break. Leave the cap slightly loosened. If you are worried about excessive evaporation of a precious solution, or of a solution with a small volume, use a permanent black marker to mark the initial fluid level on the outside of the bottle. It is always wise to autoclave pieces of glassware on a tray or a container with a low side; this simplifies clean-up if there is accidental breakage.

For autoclaving liquids in a flask, cover the opening with a loose metal cap or a cotton/gauze plug. Most commonly used, however, is a double layer of heavy duty aluminum foil, squeezed firmly, but not too tightly around the opening of the container. This double-layer foil wrap still allows adequate release of pressure that may build up in the flask during autoclaving. Single-layer foil wrap runs the risk of tearing and subsequent contamination of the contents.

It is best to sterilize liquids and dry items during separate cycles.

What Are the Time Requirements of Autoclaving?

The minimum requirements for sterilization are 15 lb of pressure per square inch at 115°C for 15 minutes of sterilization time (not including the exhaust cycle). Slightly longer sterilization times are usually not harmful and sometimes necessary. Large volumes of liquid require more sterilization time to ensure that the conditions reach the appropriate levels in the center of the liquid. Consult the media preparation staff for guidance when sterilizing unusual items.

What If the Appearance of the Indicator Tape Didn't Change during Autoclaving?

Indicator tape is often used to confirm material has been sterilized. A pale white striping or lettering on the tape turns black, indicating that that the autoclave reached the desired temperature. If the autoclaving proceeded normally but the tape didn't change color, the tape might be old, or the first two or three feet may have dried out due to improper storage. Autoclave tape should be stored in the cold and allowed to come to room temperature before use. Also remember that masking tape looks like indicator tape. If all of the above suggestions have been tested, the autoclave is not sterilizing properly.

Why Is Plastic Labware Still Wet after Applying the Dry Cycle? Is Wet Labware Sterile?

The plastic and some glassware will show condensation after a dry cycle due to the length of the cycle, or due to the cool temperature in the room that the load is brought out into. Despite the condensation, the material is still sterile. If absolute dryness is required, incubate the material, still wrapped and sealed, in a warm room for several hours to overnight. Alternatively, drying ovens set at a compatible temperature (<123°C) can dry plastic faster.

Can Your Plastic Material Be Sterilized?

Depending on the structure of the plastic, it can be sterilized by steam, gas, dry, or chemical means. The Nalgene Company catalog contains comprehensive information regarding sterilization of different plastics.

Requesting the Media Room to Sterilize Labware

Identify Your Goods

Use black permanent ink to mark your objects. The colored ink from many marker pens will wash away during autoclaving. Don't use pieces of adhesive tape to identify your labware; these will dislodge during sterilization and clog the autoclave drains.

Allow Sufficient Time

A liquid cycle (20 minutes of sterilization time) usually requires approximately 35 minutes. A wrapped (dry with fast exhaust) cycle (20 minutes of sterilization time) lasts approximately 45 minutes. On dry cycle, there is a 15 minute period beyond sterilization when the steam is exhausted, and the heat within the autoclave dries the contents. However, the actual time required to prepare your materials depends on the size of the facility, the number of autoclaves, the time of your request, the facility's workload, and the number of people on duty.

Sometimes your materials can be sterilized quickly in an emergency, but at other times you must wait your turn. Lack of planning on your part does not constitute an emergency to media room personnel with responsibilities to several laboratories. In addition autoclave cycles are pre-set, and can't be rushed.

Requesting the Media Room to Prepare Culture Media

Document Your Needs

A written request will prevent misinformation as to what was ordered and when, by whom, and when it is needed. It is crucial to place your request according to the facility's guidelines. With many media requests coming in, mistakes can happen when procedure isn't followed.

Media room personnel can provide you with information for the most commonly used media, but ultimately you should provide the details on your media needs. Does the media require a low sodium content? What pH is required? Any unusual nutrients needed?

Indicate the specific amount of media requested, and the concentration of any required antibiotic. The definition of "standard amount of antibiotic" will vary between research groups and occasionally between applications. Specify the design and size of the preferred bacteriological plate.

Allow Sufficient Time

It is best to allow at least two days for the request to be filled. File the request on Monday, the plates will be made on Tuesday, incubated overnight to test for contamination, and delivered to you on Wednesday.

Autoclaving for the Do-It-Yourselfer

Extensive rinsing and sterilization will make your washed glassware suitable for protein or DNA work. If special treatment is required, such as making the glassware endotoxin- or RNase-free, then this will require special protocols above and beyond conventional washing.

Volumes of Media and Vessels

As a general rule, add considerably less material in the container than it is designed to hold. For liquids without agar, it is safe to autoclave 900 ml in a liter bottle without losing much of the volume. The smaller the container, the safer it is to autoclave a volume of liquid approaching the maximum volume of the vessel.

The shape of the container also affects the recommended amount of media for autoclaving. For example, when autoclaving media with agar in an Erlenmeyer flask never fill the container over half of the flask volume (i.e., 500 ml in a liter flask).

Preventing Boil-Over

The most common cause of boil-over is a cap secured too tightly on a container when placed into the autoclave. The cap should be loosened at least one-half turn before sterilizing. Also don't crimp the foil too tightly around the opening. Remember to employ slow exhaust rates when autoclaving liquids.

Preventing Lumps of Powdered Stocks in Liquid or Agar Media

Never add powder or agar to a dry container. Always add some water to the container ($\frac{1}{4}$ to $\frac{1}{3}$ of the final volume of the media) before adding powdered media components. It is more accurate to do this procedure in a graduated cylinder than a flask or beaker. If your medium requires agar, do not add it yet. After your dry ingredients (except agar) are in, add a magnetic stir bar and place the container on a stir plate. This will bring most of the powder off the bottom. If the stir bar is large and the container is glass, add the stir bar carefully and gently to the container so as not to crack the bottom. It is usually not necessary to apply heat to

microbiological medium to get the powdered components into solution. If needed, place the container in a warm water bath to help dissolve the powders. Add more water until approximately 80% of the final volume of the medium is in the container. This allows a bit of volume to adjust the pH of the medium, but don't adjust the pH until all the powder components are completely dissolved. After pH adjustment, bring the solution to its final volume with water, and mix and prepare for dispensing.

If the medium is to remain as liquid, distribute it in the desired containers, leaving adequate airspace in each container as described above. If you need to add agar, follow the same principle as with preparation of the liquid medium. Never add dry agar to a dry container before adding the medium. Determine the final volume your container will hold and add about half the liquid medium to it. Add the desired amount of dry agar to the medium in the container, and use the remainder of the appropriate volume of medium to wash down the agar that has stuck to the sides of the container. If the agar powder remains on the sides of the flask, it will turn to a sticky residue during autoclaving that will never come off. The agar will not dissolve until autoclaving is completed.

Before pouring plates with the agar, gently swirl the contents to adequately mix the agar with the medium. *Warning*: Swirling a hot container immediately out of the autoclave will cause the solution to boil over, causing losses and possibly burning the handler. The agar will not sufficiently cool to solidify for quite some time, so allow the flask to cool slightly before swirling. If the plates are not to be poured immediately, the agar will stay molten in a 65°C water bath. If antibiotic is to be added, the agar must be cooled to approximately 48°C before adding the antibiotic, or else the antibiotic will be degraded.

Lumps of Agar

If you inadvertently cook the agar into a large lump in the flask, it is unusable, and must be properly disposed. After rinsing the flask with hot water to remove most of the agar, add enough undiluted bleach to cover the remaining lump and let it sit at room temperature until the lump dissolves. Large clumps of agar can clog pipes, so keep them out of the sink. Dispose of these clumps before you send the flask to be washed.

Use a Secondary Container

Glassware does "age" and can break over time during the washing and sterilization process. Bottles or flasks with liquid to

be autoclaved should be protected from the hot metal of the autoclave surface by placing them in a leak-proof container with enough water to cover the bottom of the bottle, jug, or glassware. Then, if the piece breaks, the floor of the autoclave is protected, and cleanup is simplified. Spilled agar or broth will bake on the surface of the autoclave floor, causing materials autoclaved subsequently to become covered with the residue of the spilled material.

Be aware of the way you leave the autoclave. If there is an agar spill in the autoclave, never turn the steam off to clean the floor of the autoclave, as this will cause the agar to harden in the autoclave drain. Clean the spill by pouring small amounts of hot water in the chambers to gradually and gently wash the agar down the drain. Stand away from the door as the steam develops from the hot water touching the chamber surface.

Always protect yourself adequately when handling the autoclave or recently autoclaved materials. Use protective gloves, wear a lab coat, and guard your eyes with safety glasses. Always open the autoclave door slowly, keeping your face away from the door opening. Steam escaping from a newly opened autoclave door can scald the face and damage the eyes.

Carbohydrates and Other Atypical Solutions

Some amino acids and carbohydrates can be safely autoclaved at 121°C; others cannot. The minimum temperature for autoclaving carbohydrates is 110°C. Before sterilization, read any information you can obtain regarding the product. For small volumes of solutions that will be used in analytical procedures, filtration is also an effective sterilization method. Elimination of particulate matter by centrifugation and a prefiltration step can help prevent clogging of the 0.45 or 0.22 micron filters used to remove bacteria and fungi. These filters will not remove mycoplasma or viruses, however.

Decontamination of Waste

First and foremost, don't dispose of biological materials in the normal trash; these materials must be decontaminated first. Some liquid cultures of microorganisms can be killed by addition of an iodine solution, such as Betadine™. Add enough to make your suspension turn deep brown in color. Organisms on agar plates or cells in tissue culture flasks should be autoclaved to decontaminate them. Second, determine the biohazard level of the material to be decontaminated. Consult your institutional guidelines on

what can and cannot be decontaminated at a given facility. These rules are often determined by the Occupational Health and Safety Office (OSHA), and they must be followed to the letter.

Autoclave bags are commonly used to dispose of biological materials. Orange bags contain biological materials that are thought to pose no threat to humans, but need to be decontaminated nonetheless. In most cities, waste of this type is safe for disposal in landfills after decontamination. Red autoclave bags are for biohazardous waste that could be infectious for humans or animals. *Never* put glass or other sharps in these bags. Broken glass can be decontaminated in a sealed cardboard box clearly marked with a description of the contents. Glass and metal sharps can also be disposed of and decontaminated by placing them in special plastic containers (red plastic) that are designed for this purpose. These special containers can be obtained from most large lab supply houses. Consult OSHA or the media room personnel for information on these containers.

To summarize, hazardous chemicals, radioactive waste, animal parts, and volatile substances should never be autoclaved. There are special facilities and conditions for their disposal. If you need to dispose of some of these materials, consult the media room personnel or other regulatory offices at your institution.

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6

Working Safely with Radioactive Materials

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The information within this chapter is designed as a supplement, not a replacement, to the training provided by your institutional rules and/or radiation safety officer. At the very least, there are some 10 fundamental rules to consider when working with radioactivity (Amerhsam International, 1974):

1. Understand the nature of the hazard, and get practical training.
2. Plan ahead to minimize time spent handling radioactivity.

3. Distance yourself appropriately from sources of radiation.
4. Use appropriate shielding for the radiation.
5. Contain radioactive materials in defined work areas.
6. Wear appropriate protective clothing and dosimeters.
7. Monitor the work area frequently for contamination control.
8. Follow the local rules and safe ways of working.
9. Minimize accumulation of waste and dispose of it by appropriate routes.
10. After completion of work, monitor yourself; then wash and monitor again.

LICENSING AND CERTIFICATION

Do You Need a License to Handle Radioactive Materials?

Whichever type of license is granted by the Nuclear Regulatory Commission (NRC), it tends to be a single license issued to the institution itself, to regulate its entire radioisotope usage. Separate licenses are not normally granted to the various departments or to individuals at that institution. However, everyone who works with radioactive materials at a licensed institution must be trained and approved to use the radioactive materials. Keep in mind that some states may have control over radioactive materials not controlled by the NRC. In addition, in "agreement states," the NRC requirements are regulated and controlled by a state agency.

Universities, governmental institutions, or industry are usually licensed to use radionuclides under a Type A License of Broad Scope (U.S. Nuclear Regulatory Commission Regulatory Guide, 1980). This is the most comprehensive license available to an institution. It requires that the institution have a radiation safety committee, an appointed radiation safety officer (RSO), and detailed radiation protection and training procedures. Researchers who want to use radionuclides in their work must present the proposal to the radiation safety committee and have it approved before being able to carry out the experiments.

There are other types of licenses issued by NRC or by agreement states. For example, these may be specific by-product material licenses of limited scope, specific licenses of broad scope, licenses for source or special nuclear materials, or licenses for kilocurie irradiation sources (U.S. Nuclear Regulatory Commission Regulatory Guide, 1979, 1976). By-product materials are the radionuclides that form during reactor processes. The most commonly used radionuclides, ^{32}P, ^{33}P, ^{35}S, ^{3}H, ^{14}C, and ^{125}I are all by-product materials. The licensing of by-product material is

covered in detail under Title 10, Code of Federal Regulations (CFR), Part 30, *Rules of General Applicability to Licensing of Byproduct Material* (10CFR Part 30), and 10CFR Part 33, *Specific Domestic Licenses of Broad Scope for Byproduct Material* (10CFR Part 33).

For more information, a recent publication by the NRC is now available entitled: *Consolidated Guidance about Materials Licenses. Program-Specific Guidance about Academic Research and Development, and other Licenses of Limited Scope*. Final Report U.S. Regulatory Commission, Office of Nuclear Material Safety and Safeguards. NOREG-1556, Vol. 7. M. L. Fuller, R. P. Hayes, A. S. Lodhi, G. W. Purdy, December 1999. You can also find information on the NRC Web site *www.NRC.gov*. The Atomic Energy Control Board, or AECB, governs radioactive use in Canada. Their Web site is *www.aecb-ccea.gc.ca*.

Who Do You Contact to Begin the Process of Becoming Licensed or Certified to Use Radioactivity?

If you want to use radioactivity in your research, you may need to become an authorized user at your institution. First, decide what type of isotope or isotopes will be used in your research, the application, how much material you will need, disposal methods, and for how long you will use it. Then, present this information to your radiation safety officer or radiation safety committee so that they can determine whether such radionuclide use is possible under your institution's license. If the request is approved, carry out the requirements stated on your institution's license to become an authorized user operating in an approved laboratory.

SELECTING AND ORDERING A RADIOISOTOPE

Which Radiochemical Is Most Appropriate for Your Research?

The Institution's Perspective

Your institution's license defines specific limits to the type and amount of radionuclide allowable on site (this includes on-site waste). Before determining how much material you think you'll need, find out how much you'll be allowed to have in your lab at any one time. You can then get an idea about how or if you'll need to space out the work requiring radioactivity.

Your Perspective

These are some of the most important parameters to consider when deciding which isotope to use.

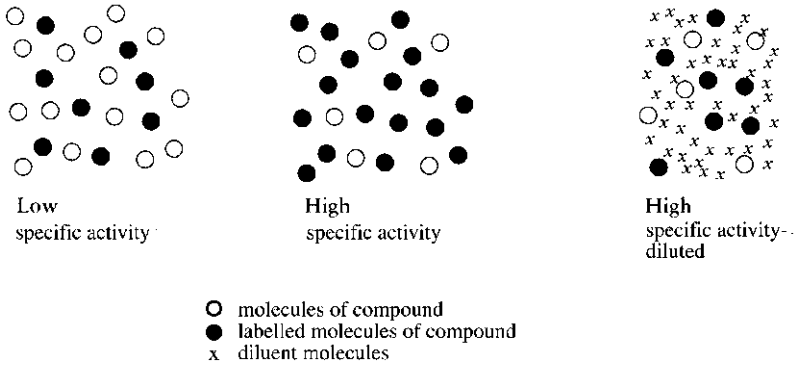

Figure 6.1 Diagrammatical representation of radiochemicals at low and high specific activity, and at high specific activity in a diluent. From *Guide to the Self-decomposition of Radiochemicals*, Amersham International, plc, 1992, Buckinghamshire, U.K. Reprinted by permission of Amersham Pharmacia Biotech.

Radionuclide, Energy, and Type of Emission (Alpha, Beta, Gamma, X ray, etc.)

In most cases you won't have the choice. You will choose the radionuclide because of its elemental properties, and its reactivity in reference to the experiment, not its type of emission. Each radionuclide has its unique emission spectrum. The spectra are important in determining how you detect the radioactivity in your samples. This is discussed more fully later in the chapter.

Specific Activity and Radioactive Concentration

The highest specific activity and the highest radioactive concentration tend to be the best since it means that there will be the greatest number of radioactive molecules in a given mass and volume (Figure 6.1). But there are two caveats to this ideal. The first is that as you increase the specific activity, you decrease the molar concentration of your desired molecule. This molecule will become the limiting reagent and possibly slow down or halt the reaction. The second danger is that at high specific activities and/or radioactive concentrations, the rate of radiolytic decomposition will increase. These parameters are discussed in more detail in Chapter 14, "Nucleic Acid Hybridization."

To take an example, a standard random priming labeling reaction requires 50 μCi (1.85 MBq)* of ^{32}P dNTP (Feinberg

**In the United States the unit of activity of "Curie" is still used. The unit of common usage is the Becquerel (Bq). Whereas 1 Curie = 3.7 × 10^{10} disintegrations per second (dps), the Bq = 1 dps. For example, to convert picocuries (10^{-12} Curies) to*

and Vogelstein, 1983). At a specific activity of 3000 Ci/mmol, that 50 μCi translates to 16.6 femtomoles of ^{32}P dNTP being added to the reaction mix, while 50 μCi of a ^{32}P labeled dNTP at a specific activity of 6000 Ci/mmol will add only 8.3 femtomoles to the reaction. Unless sufficient unlabeled dNTP is added, the lower mass of the hotter dNTP solution added might end up slowing the random prime reaction down, giving the resulting probe a lower specific activity than the probe that used the 3000 Ci/mmol material.

Label Location on the Compound

Consider the reason for using a radioactive molecule. Is the reaction involved in the transferring of the radioactive moiety to a biomolecule, such as a nucleic acid, peptide, or protein? Is the *in vivo* catabolism of the molecule being studied, perhaps in an ADME (absorption, distribution, metabolism, and excretion) study? Or perhaps the labeled molecule is simply being used as a tracer. For any situation, it's worthwhile to consider the following impacts of the label location: First, will the label's location allow the label or the labeled ligand to be incorporated? Next, once incorporated, will it produce the desired result or an unwanted effect? For example, will the label's presence in a nucleic acid probe interfere with the probe's ability to hybridize to its target DNA? The latter issue is also discussed in greater detail in Chapter 14, "Nucleic Acid Hybridization."

There are some reactions where the location of label is not critical. A thymidine uptake assay is one such case. The labeling will work just as effectively whether the tritium is on the methyl group or on the ring.

The Form and Quantity of the Radioligand

The radionuclide is usually available in different solvents. The two main concerns are the effect (if any) of the solvent on the reaction or assay, and whether the radioactive material will be used quickly or over a long period. For example, a radiolabeled compound supplied in benzene or toluene cannot be added directly to cells or to an enzyme reaction without destroying the biological systems; it must be dried down and brought up in a compatible solvent. Likewise a compound shipped in simple aqueous solvent might be added directly to the reaction, but might not be

Becquerels, divide by 27 (27.027): 50 μCi = 50 $\times$ 10^{-6} Ci = (50 $\times$ 10^{-6} Ci $\times$ 3.7 $\times$ 10^{10} dps/Ci) = 1.85 $\times$ 10^{6} dps = 1.85 $\times$ 10^{6} Bq = 1.85 MBq.)

the best solvent for long-term storage. From a manufacturing perspective, the radiochemical is supplied in a solvent that is a compromise between the stability and solubility of the compound and the investigator's convenience.

Some common solvents to consider, and the reasons they are used:

- *Ethanol, 2%*. Added to aqueous solvents where it acts as a free radical scavenger and will extend the shelf life of the radiolabeled compound.
- *Toluene or benzene*. Most often used to increase stability of the radiolabeled compound, and increase solubility of nonpolar compounds, such as lipids.
- *2-mercaptoethanol, 5 mM*. Helps to minimize the release of radioactive sulfur from amino acids and nucleotides in the form of sulfoxides and other volatile molecules.
- *Colored dyes*. Added for the investigator's convenience to visualize the presence of the radioactivity.

When not in use, the "stock" solution of the radioactive compound is capped and usually refrigerated to minimize volatilization/evaporation.

What Quantity of Radioactivity Should You Purchase?

There are three things to consider when deciding how much material to purchase:

1. How much activity (radioactivity) will be used and over what period?
2. What are the institutional limits affecting the amounts of radioisotope chosen that your lab may be authorized to use?
3. What are the decomposition rate of your radiolabeled compound and its half-life.

In general you will want to purchase as large a quantity as possible to save on initial cost, while at the same time not compromising the quality of the results of the research by using decomposed material. For example, certain forms of tritiated thymidine can have radiolytic decomposition rates (thymidine degradation) of 4% per week. This decomposition rate is not to be confused with tritium's decay rate, or half-life, which is over 12 years. Stocking up on such rapidly decomposing material, or by using it for more than just a few months could compromise experiments carried out later in the product's life.

When Should You Order the Material?

Analysis Date

Ideally you will want to schedule your experiments and your radiochemical shipments such that the material arrives at its maximum level of activity and lowest level of decomposition. This will tend to be when the product is newer, or nearer its analysis date (the date on which the compound passes quality control tests and is diluted appropriately so that the radioactive concentration and specific activity will be as those stated on the reference date). Some isotopes and radiochemicals decompose slowly, so it is not always necessary to take this suggestion to the extreme. As you use a radiolabeled product, you'll come to know how long you can use it in your work. An ^{125}I labeled ligand will not last as long as a ^{14}C labeled sugar. An inorganic radiolabeled compound, such as Na^{125}I or sodium ^{51}chromate, will decompose at the isotope's rate of decay, whereas a labeled organic compound, such as the tritiated thymidine alluded to earlier, will decompose at a much faster rate than the half-life of the isotope would indicate. Manufacturers take this into account by having a terminal sale date. The material will only be sold for so long before it is removed from its stores. Up until this date you will be able to purchase the material and still expect to use it over a reasonable period of time.

Reference Date

The reference date is the day on which you will have the stated amount of material. If you purchased a 1 mCi vial of ^{32}P dCTP, you will have greater than 1 mCi (37 MBq) prior to the reference date, 1 mCi on that date, and successively less beyond the reference date. (Note that since you will most likely receive your radioactive material prior to reference, it is possible to exceed possession limits; consider this when determining limits on your radiation license.) In the case of longer-lived radioisotopes, such as ^{3}H and ^{14}C, the analysis date will also serve as the reference date.

How Do You Calculate the Amount of Remaining Radiolabel?

The most straightforward way of calculating radioactive decay is to use the following exponential decay equation. For convenience's sake, most manufacturers of radiochemicals provide decay charts in their catalogs for commonly used isotopes. This equation comes in handy for the less common isotopes.

$$A = A_0 e^{-0.693t/T}$$

where

A_0 is the radioactivity at reference date,
t is the time between reference date and the time you are calculating for,
T is the half-life of the isotope (note that both t and T must have the same units of time).

It is easy to use the aforementioned decay charts as shown in the following two examples.

Say you had 250 μCi of ^{35}S methionine at a certain reference date, and the radioactive concentration was 15 mCi/ml. Now it is 25 days after that reference date. You calculate your new radioactive concentration and total activity in the vial by looking on the chart to locate the fraction under the column and row that corresponds to 25 days postreference. This number should be 0.820. Multiply your starting radioactive concentration by this fraction to obtain the new radioactive concentration:

$$15\,\text{mCi/ml} \times 0.820 = 12.3\,\text{mCi/ml}$$

The total amount of activity can be likewise calculated for ^{35}S with a half-life of 87.4 days; namely $A = A_0 e^{-0.693t/T} = 15\exp(-0.693 \times 25/87.4) = 12.3$ mCi/ml.

For the second example you can find out how much activity you had before the reference date. Some decay charts only have postreference fractions, but if you have a 1 mCi vial of ^{33}P dUTP at 10 mCi/ml, and it is 5 days prior to the reference date, how do you figure out how much you have? Go to the column and row on the ^{33}P decay chart corresponding to 5 days postreference. There you will see the fraction 0.872. You will divide your reference activity and radioactive concentration by this number to obtain the proper amount of activity present, or 1/0.872 = 1.15 mCi. Note that the values should be greater than the stated amounts of activity and the referenced radioactive concentration. For the calculation method you are now looking for A_0. Therefore $A_0 = Ae^{0.693t/T} = 10\exp(0.693 \times 5/25) = 11.5$ mCi/ml, using a half-life of 25 days for ^{33}P.

How Long after the Reference Date Can You Use Your Material?

Radioactively labeled compounds do not suddenly go bad after the reference date. It isn't an expiration date. It is used as a benchmark by which you can anchor your decay calculations as described above.

Table 6.1 Shelf Lives for Commonly Used Isotopes

^{32}Phosphorous	1–3 weeks
^{33}Phosphorous	4–12 weeks
^{35}Sulfur	2–6 weeks
^{125}Iodine	3–12 weeks
^{3}Hydrogen	1–12 months
^{14}Carbon	1–2 years

Only you can determine how long you can use your radioisotope after the reference date. The answer depends on the isotope, the compound it's bound to, the experiment, storage, the formulation of the product, and the like. Table 6.1 lists the general ranges for the most commonly used radioisotopes, which is a guideline only. As you carry out your work, you will discover when your material starts to give poorer results.

Can You Compensate by Adding More Radiochemical If the Reference Date Has Long Passed?

Sometimes it is not that simple. As an example of the complexities involved with radiolytic decomposition, suppose you had a vial of ^{32}P gamma labeled ATP that you routinely use to label the 5′ end of DNA via T4 Polynucleotide kinase. If one half-life has passed since the reference date (14.28 days), you will have 50% of the stated radioactivity remaining. You might still achieve satisfactory 5′ end labeling with T4 Polynucleotide kinase if you double the amount of the ^{32}P added to the reaction. Often, however, you may find that though you have compensated for the radioactive decay by adding more material, you have also introduced more of the decomposition products, which will be fragments of the original labeled compound and free radicals. You also will have added more of the solute that might be present in the stock vial. These contaminants and decomposition products can significantly interfere with the reaction mechanism and compromise your results.

HANDLING RADIOACTIVE SHIPMENTS

What Should You Do with the Radioactive Shipment When It Arrives?

The radiation safety officer is responsible for ensuring that radioactive materials are received in satisfactory condition, but

procedures may vary within the institution. Sometimes the RSO will check the shipping box for contamination and then discard the outer box, forwarding only the radioactive container to the researcher. At other facilities the receiving group will do a wipe test on the outer shipping container only, and if found to be uncontaminated, forward the entire package to the researcher. Upon receipt, you will want to carry out a final wipe test on the vial of radioactive material before opening, to make sure there is no gross contamination.

The Wipe Test

The manner in which a wipe test is to be carried out will be described in your institution's radioactive use license, the details of which can be explained to you by your RSO. A wipe test involves dragging or rubbing a piece of absorbent paper, or cotton swab across a portion of a vial, package, or surface (the standard area being 100 cm^2). You are testing for the presence of removable radioactive contamination. The paper or swab may be dry or wetted with methanol or water. Your RSO will let you know which way is preferred by the institution. After wiping the surface, the paper or swab may be placed into a liquid scintillation counter (LSC) to detect if any contamination was removed. It is usually best to count the wipes in an LSC rather than use a count-rate meter because some isotopes are not detected with a count-rate meter (e.g., tritium). Knowing the radioisotope and its decay products will help to determine the best detection method. The count-rate meter will be described more fully below.

A Wipe Test Detected Radioactivity on the Outside of the Vial. Does This Indicate a Problem?

If you detect contamination on the outside of your vial, contact your RSO. She will tell you, based on experience and institutional norms, whether the amount of contamination you have found is of concern, and whether the counts detected on the LSC may be artifactual (caused by chemiluminescence), or if they are being caused by radioactive contamination.

While the ideal is to have no detectable counts on the outside of the primary container, the act of packaging, shipping, and handling can work together to make this difficult to achieve. Then there are some radioisotopes, most notably, ^{35}S and ^{3}H, that are volatile, and can leach through the crimped overseals. This is one of the reasons why radioactive materials are shipped in secondary

containers. The vial of ^{35}S labeled methionine you might receive is first dispensed into a primary container, which is sealed, then placed into a secondary container. The secondary container usually has absorbent material placed in it that will absorb any liquid should there be a spill. It is always prudent to wear some thin plastic gloves when dealing with radioactive materials, especially when they first arrive and you have no indication on whether or not they are contaminated.

The NRC has set action limits to contaminated surfaces of outer packages and containers, and the RSO is required to contact the NRC when these levels are surpassed. The amount of contamination considered significant will differ, depending on whether the activity is on the primary container, secondary container, or the outer package. Based on these action levels, an institution sometimes sets its own, lower, contamination limits. Contact your RSO for more information on contamination limits.

Your Count-Rate Meter Detects Radiation on the Outside of a Box Containing 1 mCi of a ^{32}P Labeled dATP. Is It Contaminated?

When you put a count-rate meter up to the outer package containing the ^{32}P substance, you will hear clicking sounds, indicating the presence of radioactivity. To determine whether the radiation is coming from contamination on the outside of the package, or emanating from the vial of material, it is necessary to carry out a wipe test on the package. In the overwhelming majority of cases, what the instrument is detecting is called *Bremsstrahlung*.

Bremsstrahlung

Bremsstrahlung, or "braking radiation," is created when a beta particle interacts with the shielding material to produce X rays. The Plexiglas™ vial that contains the radioactive material is sufficient to block essentially all beta emissions but not the X rays. Is *Bremsstrahlung* dangerous? The dose rate detected on the surface of a vial with 1 mCi of ^{32}P tends to be between 1 and 5 mrem/h, while there will be no detectable dose rate three feet away. This level of dose rate is considered low for those working in occupations that use radioactivity. It is important to remember that dose rate decreases as you move away from the source. Doubling the distance from the source will quarter the radiation dose. This is known as the inverse square law, and it is applicable whenever the source can be considered a point source. You may wish to discuss dose rates in more detail with your RSO.

You Received 250 μCi of ^{32}P and the Box Wasn't Labeled Radioactive. Isn't This a Dangerous Mistake?

Both the Department of Transportation (DOT) and The International Air Transport Association (IATA) have regulations concerning the labeling of packages containing limited quantities of radioactive materials (International Air Transport Association [IATA] Dangerous Goods Regulations, 6.2, and Code of Federal Regulations [CFR] 173.421, 173.422, 173.424, and 173.427). A package is defined as containing a limited quantity of an isotope if it conforms both to a certain physical amount of radioactive substance, and if the dose rate on the outside surface of the package is less than 0.5 mrem/h. For example, the isotope ^{32}P has a limited quantity of 3.0 mCi if it is in liquid form. This means that if the package contains less than 3.0 mCi, and if the dose rate is less than 0.5 mrem/h on the package's surface, then it is considered to be a package of limited quantity. So the package does not require an external label which bears the marking "radioactive." The regulations do require, however, that there be such labeling somewhere inside of the package, and that the packaging itself prevent leakage of the radioactive material under "conditions likely to be encountered during routine transport (incident-free conditions). . . ." (International Air Transport Association [IATA] Dangerous Goods Regulations, 6.2).

DESIGNING YOUR EXPERIMENTS

How Do You Determine the Molarity and Mass in the Vial of Material?

Let's start with some definitions.

Specific Activity

The definition of specific activity is the amount of radioactivity per unit mass, and is usually reported as curies per millimole (or Becquerels per millimole), abbreviated Ci/mmol (Bq/mmol). Specific activity is a quantitative description for how many molecules in a sample are radioactively labeled.

In order to determine the ratio of labeled molecules in the total molecule population, the specific activity of the material is divided by the theoretical maximum specific activity. The theoretical maximum specific activity is defined as the greatest amount of radioactivity that can be achieved if there were 100% isotopic abundance at a single location. This number is specific to the type of radionuclide.

As a simple example, the theoretical maximum specific activity for ^{32}P is 9131 Ci/mmol. If the percentage of radioactive molecules in a 3000 Ci/mmol product is desired, it simply is a matter of dividing 3000 by 9131 to find that approximately one-third of the molecules in that sample are radioactive. This will give the investigator an idea of how many radioactive molecules may get incorporated into the final product.

Molarity

The molarity of a labeled compound in solution can be calculated by dividing the radioactive concentration of your radiochemical by its specific activity:

$$\frac{\text{Radioactive concentration}}{\text{specific activity}}$$

For example, a vial of ^{32}P-labeled gamma ATP at a radioactive concentration of 10 mCi/ml and specific activity of 3000 Ci/mmol will have a molarity of

$$\frac{10\,\text{mCi}/\text{ml}}{3000\,\text{Ci}/\text{mmol}} = 3.33\,\mu\text{M}$$

Moles

Once you have the molarity of your stock solution, simply multiply that by the volume of stock you'll be adding to your reaction in order to obtain the number of moles you have

$$\text{Molarity} \times \text{volume} = \text{moles}$$

Continuing with this example, if you are adding 5 μl of the ^{32}P-ATP to your reaction, you will be adding

$$3.33\,\text{pmol}/\mu\text{l} \times 5\,\mu\text{l} = 16.7\,\text{pmols}$$

to the reaction vessel. After calculating the molarity of your radioactive stock solution, you might be shocked to learn how low it is. You might even think that it's too low for the reaction to run, or perhaps your protocol states that you should start out with a higher molarity. The solution is as follows:

Make up a stock solution of the cold compound in question, at the appropriately higher molarity. To this stock or to the reaction mix itself, add the amount of radioactivity required. The number of picomoles of radiolabeled compound that you'll be adding to your reaction mix will, in most cases, be so low that

it will make no practical difference to the overall molarity of the compound. Note, however, that by adding cold compound, you will be dramatically lowering the specific activity of the radioactive label, which is in the reaction vessel. You may find that you get lower incorporation rates.

How Do You Quantitate the Amount of Radioactivity for Your Reaction?

DPM, CPM, and μCi

One curie of activity is 3.7×10^{10} disintegrations per second (dps) or 2.22×10^{12} disintegrations per minute (dpm). Thus the definition of a μCi is 2.22×10^{6} dpm, regardless of the isotope involved. Disintegrations per minute (dpm) are a function of nature; counts per minute (cpm) are a function of a detection device. Cpm/dpm is a measure of the instrument's efficiency to detect an isotope's decay event. A liquid scintillation counter (LSC), because of its photomultiplier tubes, cannot be 100% efficient. The instrument will give values in cpm, which will always be lower than the true number of disintegrations occurring in the sample. This counting efficiency can vary with the isotope and even the type of solvent and scintillation fluid (if a liquid scintillation method is used) that your samples are in. The counting efficiency should be determined if quantitative results are needed in your work. The procedure is to measure the cpm detected from a sealed calibration source that contains a known number of dpm. The percent efficiency of your counter is calculated by dividing the counted cpm by the dpm as indicated on the vial, then multiplying this quotient by 100. Typical examples of counting efficiencies for some commonly used isotopes are 75 to 85% for ^{14}C, ^{35}S, and ^{33}P; 35 to 55% for ^{3}H; 70 to 80% for ^{125}I (this radioisotope, while being a gamma ray emitter, is actually more efficiently counted on an LSC); and almost 100% for ^{32}P. These efficiencies are approximations only. Efficiencies can vary widely, however, depending on instrument, isotope, and sample type.

The Becquerel (Bq)

A Becquerel, or Bq, is a *Systeme Internationale* unit of measure for radioactivity. One Bq is one disintegration per second (dps). One dpm will be 60 Bq. One μCi is defined as 37 kBq. The Bq is a defined value of radioactivity that is small, whereas the Ci is very large. In the United States, the Ci is still a common unit. Most other countries have converted to using the Bq.

STORING RADIOACTIVE MATERIALS

As you gain experience with your radioactive materials, you will gain insight into two of their important but not intuitive physical properties. First, their lifetime is shorter than their unlabeled counterparts (because attached to them is a huge ball of energy waiting to blow). Second, the compound's shelf life is often dramatically less than the half-life of the isotope used to label it.

What Causes the Degradation of a Radiochemical?

The mechanisms of radiolytic decomposition are fairly complex but can be divided into primary and secondary decomposition (Amersham International, 1992). Internal primary degradation is caused by the release of energy from the radioactive atom's unstable nucleus. This energy release in turn is thought to break up the bonds of the parent molecule, destroying it (for very large molecules, e.g., proteins, it is unlikely to destroy the entire molecule). The rate of primary degradation is identical to the radioactive decay rate.

Another mode of primary decomposition is external, arising when ionizing radiation emissions hit nearby molecules. The energy transferred to the molecule is often enough to break chemical bonds within the molecule producing random fragments.

Secondary decomposition is caused by free radicals generated by the interaction of beta particles with the solvent. It is the most insidious form of decomposition. Free radicals can potentially interact with any compound within the solvent, generating innumerable contaminants and breakdown products. Some reactions generate more free radicals, leading to exponential rates of breakdown and contaminant production.

What Can You Do to Maximize the Lifetime and Potency of a Radiochemical?

1. Do not alter the recommended storage conditions.

Colder is not always better. If the solvent containing the radioactive material is stored at a temperature that allows the solvent to freeze slowly, an event called molecular clustering will occur (Figure 6.2). The freezing solvent pushes nonsolvent molecules into pockets or clusters. This results in extremely high radioactive concentrations, which in turn will cause extremely high rates of radiolytic decomposition. Examples of solvents freezing slowly will be: water at –20°C, or ethanol at –70°C. If the solution is quick-frozen (in liquid nitrogen), you will avoid the effects of molecular clustering.

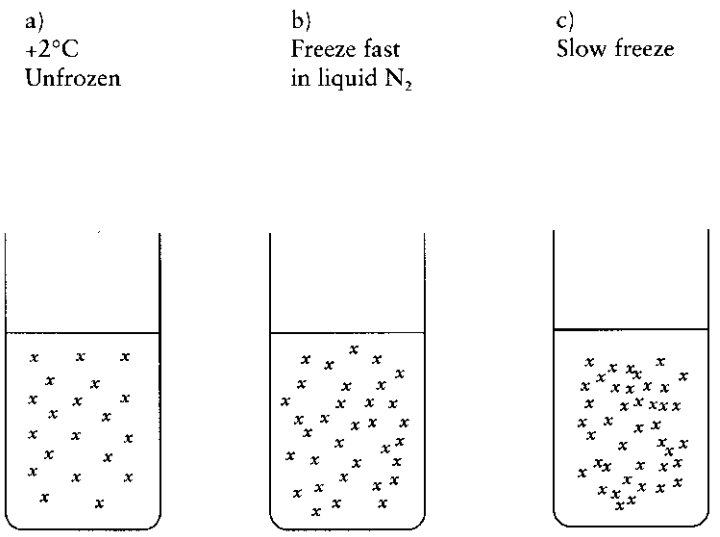

Figure 6.2 Molecular clustering effects. From *Guide to the Self-decomposition of Radiochemicals*, Amersham International, plc, 1992, Buckinghamshire, U.K. Reprinted by permission of Amersham Pharmacia Biotech.

2. Keep the radioactive concentration as low as possible to minimize primary external and secondary decomposition.
3. Minimize the number of freeze-thaws, which may increase the decomposition rate
4. Don't alter the recommended solvent.

Some solvents will cause greater rates of radiolytic decomposition. It cannot be predicted which ones will be better or worse until they have been tested. Manufacturers will have chosen the most appropriate solvent for the radioactive compound.

5. Schedule experiments to consume your store of radioisotope as quickly as possible.

What Is the Stability of a Radiolabeled Protein or Nucleic Acid?

After labeling or incorporation of radioactivity into your molecule of interest, radiolytic decomposition occurs. As the isotope decays into the surrounding solution, there will be primary decomposition, giving rise to nicked, or broken strands in your labeled nucleic acid, as well as the less predictable secondary decomposition, which might break the chemical bonds comprising that molecule. It is best to use your labeled molecule as soon as possible, or to store in as dilute a concentration as is reasonable for your work.

In this regard, ^{32}P is the most offending of the three most commonly used radioisotopes (^{32}P, ^{33}P, and ^{35}S). Compounds

labeled with ^{32}P can have extremely high specific activities, and the energy of the beta is also extremely high. On the other hand, ^{33}P and ^{35}S have similar energies to each other. They have much lower emission energies and thus are less destructive to surrounding molecules. This issue is discussed in greater depth in Chapter 14, "Nucleic Acid Hybridization."

Radioactive Waste: What Are Your Options and Obligations?

It is essential to keep accurate records of the amount and type of radioactive waste that you generate. The RSO keeps track of all incoming radioactivity and all outgoing radioactive waste, so it is important to keep track of the material you use, store, and dispose of for the RSO's records. When the NRC or governing body inspects your institution, it will check its receipt and disposal records. If they are not in order, there is the possibility of suspension of your institution's license.

Obligations

Consult your RSO. Your institution has in place a detailed waste management program, and your radiation license requires that you follow your institution's waste handling procedure without variance. Minimally you must separate the waste of different nuclides, and you will probably be required to separate liquid and solid wastes and to minimize the creation of mixed waste, which is discussed further below.

Options

Generate no more radioactive waste than is absolutely necessary. Most countries have few sites that accept radioactive waste, so the costs per pound are outrageously expensive, forcing more institutions to store waste locally. Although there have been major advances in radioactive waste processing, these new technologies may be years away from being commonly available to any but the largest producers of radioactive waste.

Radioactive waste can be treated as nonradioactive after 10 half-lives. This is convenient for isotopes with short half-lives, such as ^{32}P and ^{33}P (10 half-lives is 250 days for ^{33}P, 143 days for ^{32}P), but the very long half-lives of ^{14}C (5730 years) and ^{3}H (12.41 years) more urgently illustrate the need for waste reduction. Your institution will have a policy on which radioisotopes will be disposed of by "decay in storage" or dumping into the sanitary sewer.

Limit the production of mixed waste, which is defined as a combination of two or more hazardous compounds, such as scintilla-

tion fluid and radioactivity. This waste is especially expensive to process and it will be worth your time to investigate if this type of waste can be avoided or minimized.

HANDLING RADIOACTIVITY: ACHIEVING MINIMUM DOSE

How Is Radioactive Exposure Quantified and What Are the Allowable Doses?

Radiation exposure is defined in REM, or "radiation equivalent man," and mrem, or millirem. In the United States, the maximum annual allowable dose is 5000 mrem to the internal organs, and 50,000 mrem to the extremities for those individuals working with radioactivity. (Note: Most other countries use a similar level, but the units are the international units of Sieverts, 5000 mrem = 50 mSv.) For comparison, the average person who doesn't work with radioactivity receives between 300 and 500 mrem per year. They receive this exposure from sources such as ^{40}K (potassium-40) and other naturally occurring radioactive isotopes found in foods, soil and rock, radon gas, cosmic rays, medical and dental X rays, and so forth.

Monitoring Technology: What's the Difference between a Count-Rate Counter and a Dose-Rate Meter?

Count-Rate Meter

A count-rate meter, generally configured with a Geiger-Müller detector, is used to detect small amounts of surface contamination. It is a common laboratory instrument. The unit is small and hand-held with an attached probe. When the probe face is directed toward an appropriate radiation field (most beta or gamma emitters), the count-rate meter produces the familiar clicking sound made so famous in science fiction movies of the 1950s. On the body of the meter is either an analog needle-type gauge or a digital readout, which will indicate the counts per second (cps) or counts per minute (cpm) of the field based on where the probe is located. In general, the efficiency of the count-rate meter versus a liquid scintillation counter, for example, is quite low. It has been designed to provide a quick, qualitative means of determining the presence of minute quantities of radioactivity (most instruments will detect between 50 and 5000 cps).

In the presence of a significant radiation field, the count-rate meter will be overloaded and cease to "click" or give a reading on the needle gauge. You can misinterpret the lack of sound

as meaning that no dose field is present, when in fact what you need is another type of instrument to detect dose rates. The count-rate meter is best used to detect nanocurie or less quantities of contamination on gloves, benchtop, and other equipment.

Dose-Rate Meters

The dose-rate instrument should be used to detect larger quantities of ionizing radiation. It measures radiation fields in units of mrem/h. The dose rate meter also has a probe, generally an ionization chamber, and registers values on an analog needle gauge, or digital reading. A dose-rate meter does not aurally indicate the presence of radioactivity with clicks, however. It converts detected nuclear events into units that can be related to how much radioactive dose is present. This conversion is dependent on type and energy of emission, as well as on the distance from the radiation source. A count-rate meter detects an event, while a dose-rate meter converts that event into a meaningful energy reading. It is not a simple matter to convert cpm into a dose rate of mrem/h in our heads or by use of a chart because of the number of variables involved. Where a count-rate meter will go off scale, or become overloaded in a modest radiation field of 10 mrem/h, a dose-rate meter can measure much greater readings, depending on the particular instrument. Dose-rate meters are generally more expensive and not normally present in a lab, but your RSO will have them on hand when one is needed.

An illustration of the difference between dose rate and counts per second (or per minute) is seen in the following example. If you place a 1 mCi vial of ^{32}P-labeled dCTP right next to the probe face of a count-rate meter, there would suddenly be an "alarming" clicking sound. If you were to then open the vial, face the probe vertically down toward the open solution of ^{32}P, the meter would almost immediately become overloaded and stop giving off any sound, and fail to register a value on the needle gauge (The author does not recommend that you actually do this as it will needlessly increase both your exposure and the editor's legal liability.)

If you were to place the same sealed vial directly next to a dose-rate meter, you will detect an exposure rate of 2 to 5 mrem/h, which is typically considered to be a low or modest exposure dose field. If the dose-rate meter's probe is one inch directly above the open vial, you will read in excess of 1.0 rem/h, or 1000 mrem/h, a dramatic increase in dose. This is a significantly higher dose

rate, yet the count-rate meter would not provide you with any warning.

To relate this scenario into the amount of exposure a dose film badge or thermoluminescent dosimeter (TLD) used for personnel monitoring might detect, suppose that your hand with a finger badge were placed directly over the open vial for one minute. Your finger might receive close to 17 mrem, which can quickly add up if it is part of your routine. On the other hand, if you held the closed vial in your hand for one hour, the finger dosimeter would register only 5 mrem, or 0.01% of the annual allowable dose.

What Are the Elements of a Good Overall Monitoring Strategy?

Identify the Hot Spots

Consider inviting your RSO to inspect the organization of your radioactive work area and to monitor your laboratory with a dose-rate meter to identify locations of significant exposure. This step is especially relevant when working with strong emitters such as ^{32}P and ^{125}I.

Short Term, or Contamination Monitoring

At the start of each workday, use a count-rate meter to check any work surface you plan to encounter, such as the benchtop and the lip of the hood. Next apply a count-rate meter to monitor the entire front part of your body and legs; pay special attention to your gloves and lab coat, especially the sleeves.

In all cases of contamination of yourself or if a serious spill occurs, your institution will have a very clear procedure on what steps to take to resolve it. You must know this procedure before working with radioactivity in your lab.

Long Term, or Dose Monitoring

Whole body dosimeters, often referred to as "badges" should be worn on the chest or abdomen to estimate exposure to critical organs. Ring badges worn on fingers are recommended to monitor extremity exposure. In some cases, and with particular radioisotopes in use, the radiation safety officer may require more specific monitoring techniques in order to test for the presence of radioactive contamination. A common example is the requirement of urine samples from those investigators working with tritium, and thyroid monitoring for those working with radioactive iodine.

What Can You Do to Achieve Minimum Radioactive Dose?

Attitude

Consider the benefits of an attitude whereby everyone working with radioactivity continuously ponders if they are working in the safest, most efficient manner. Is a particular radioactive experiment necessary? Can the amount of radioactivity in an experiment be reduced? Is there a faster, safer way to carry out the work? Questions like these will reduce cost and radioactive exposure. An institution's RSO is also required to implement a continuing education program regarding the principles of keeping personnel exposure dose low.

If you find yourself becoming stressed while handling radioactivity, or if that "incessant clicking sound" of the count-rate meter is causing a heightened sense of alarm, you can always step away from the bench to put things into perspective. Estimate how much dose you are receiving from your activities and relate those back to your annual allowable dose.

Time

Work quickly and neatly. In the example above, a finger lingering for 1 minute over an open 1 mCi vial of ^{32}P will receive 17 mrem, whereas a 10 second exposure receives a sixfold lower dose.

Practicing the manipulations of your experiments with non-radioactive materials will identify problem areas and ultimately enable you to work faster and safer. Working with radioactivity while feeling panicked or rushed will slow you down or cause an accident. If you can't smoothly do the motion in 10 seconds, take 20. You'll improve through time and all along will be well aware of your estimated dose. You'll automatically be striving to lower your dose.

Distance

Dosage decreases with distance. Why? A radiation source is like a light bulb. As the rays radiate outward in a sphere, they cover a wider area but become less potent at any single point. Use the inverse square law to your advantage. Can you pipette with a longer pipettor? Can you place the reaction vial even a few inches farther from you and others in the lab? Can you place a film cassette containing a radioactive membrane farther away from your work area? Small steps such as these can go a long way in reducing dose to you and your colleagues.

Shielding

A good shielding strategy will effectively reduce dose rate without preventing you from working smoothly and safely. It will not force you to get closer or stay longer in high radiation areas. If the use of lead-lined gloves makes you feel like you're working in a vat of honey and increase the likelihood of a spill, you might want to consider alternative shielding.

Shielding for Beta Emitters

Acrylic plastic (Plexiglas™) is used for the pure beta emitters, like ^{32}P, ^{33}P, and ^{35}S. A half inch thick piece of acrylic will stop essentially 100% of all betas, even for strong emitters, such as ^{32}P.

Shielding for Gamma Emitters

Lead will attenuate rather than completely obstruct gamma or X radiation. You may see in some literature that for a particular gamma-emitting isotope, a certain thickness of lead is required to "reduce the dose rate by a factor of 10." This means that if a source is giving off a field of 100 mrem/h without shielding, the dose rate with that particular thickness of lead will be brought down to 10 mrem/h. For example, ^{125}I needs to have 0.25 mm of lead shielding in order to reduce the dose rate by a factor of 10. Each successive layer of 0.25 mm will continue to decrease the dose rate by a factor of 10.

Lead is best used as shielding for an isotope giving off both gamma radiation and beta particles, rather than a combination of acrylic and lead.

Volatile Nuclides

The three isotopes you are likely to encounter with volatile properties are ^{3}H, ^{35}S, and ^{125}I. Their chemical properties and the incredibly complex reactions involved with radiolytic decay cause these two isotopes to form gaseous by-products. If you work with any of these isotopes, your RSO and institution may have approved fume hoods for their use.

Isotopes That Do Not Require Shielding

Tritium, being a very weak beta emitter, travels only a few microns in air. Acrylic shielding would be of no use. What you do not want to do is to ingest tritium. Tritium in an aqueous form is 25,000 times more radiotoxic than tritium in a gaseous form.

How Can You Organize Your Work Area to Minimize Your Exposure to Radioactivity?

If feasible, select bench space at the corner of the room, rather than in a central location, to reduce unnecessary traffic. Clearly delineate this work area as radioactive. Although it is not always possible due to space restrictions, it is recommended that if your lab is working with different radioisotopes that there be separate work areas for each radioisotope. Check with your RSO about any additional requirements listed on the institution's license.

Of main importance will be arranging your work space. Begin with absorbent material, perhaps a double thick section, taped onto the bench. A waste container that shields against the radioactivity should be placed in a location that makes it easy, quick, and safe to dispose of pipette tips, hot gloves, and the like. A box made of acrylic with a lid is sufficient for ^{32}P, ^{33}P, and ^{35}S, while for ^{125}I, lead-impregnated acrylic will help attenuate the gamma rays. Each radioisotope may need its own separate container for waste, depending on your institution's disposal protocols.

If you are using ^{32}P, acrylic shielding between you and the source is strongly recommended. There are many commercially available shields that will meet your needs. Once you establish your radioactive area, do a couple of practice runs to make sure that your work area is properly organized. Bring the RSO in so that she/he can approve your radioactive area and perhaps make further suggestions.

You'll want to examine closely any areas or actions that have the potential for high doses. An open vial of ^{32}P, an Eppendorf tube with 50 μl of ^{32}P, and a tray containing your blot with hybridization solution mixed with radioactive probe will all be obvious areas where you'll need to pay close attention. The open vial may simply need an acrylic pipette guard on your pipettor in order to bring the dose down 10,000 fold. The Eppendorf incubation tube can be kept in an acrylic box, or behind acrylic shielding while the labeling reaction is going on. You may devise a way of not picking the reaction tube up with your fingers while you remove the reaction mix with a pipettor. The blotting container may present a potential spill. Finding a safe, out-of-the-way place, preferably in a fume hood and behind some acrylic shielding will go a long way toward reducing dose.

How Can You Concentrate a Radioactive Solution?

Three convenient approaches are lyophilization, a spinning vacuum chamber, and drying with a gentle stream of nitrogen gas.

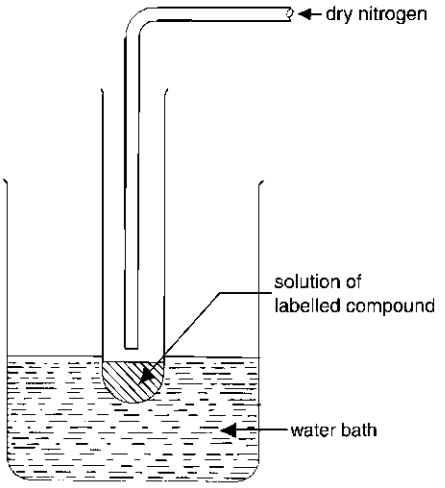

Figure 6.3 Removal of solvent from a non-volatile radiochemical using dry nitrogen. From *Guide to Working Safely with Radiolabelled Compounds*, Amerhsam International, plc, 1974, Buckinghamshire, U.K. Reprinted by permission of Amersham Pharmacia Biotech.

There is significant risk of contamination when using a lyophilizer or spinning vacuum chamber, so most facilities dedicate specific equipment for radioactive work. Blowing a very gentle stream of nitrogen gas over the solution works efficiently, but practice is required to avoid blowing the radioactive solution out of its container.

The nitrogen stream method is straightforward (Figure 6.3). Attach a small glass pipette/dropper tip to tubing that is attached to the gas regulator of a tank of dry nitrogen gas, being careful not to break the top of the pipette into your hand. Turn on the gas flow, keeping the gas flow as gentle as possible. This procedure requires very little nitrogen flow. Before impinging upon the surface of the radioactive material, test the gas flow on a vial containing a like amount of water. Adjust the flow so that there is no splashing of the liquid but only a noticeable indentation of the liquid's surface. Once you are satisfied that it is safe, gently direct the stream of gas onto the surface of the radioactive liquid, ensuring no splashing. Do all of this in a hood and in a location that is safe and will be able to contain accidental spills.

Continue blowing off the solution until dryness. Overdrying can sometimes be of concern, so it is best not to leave the area and come back to it after an extended period. It is also best to bring the solution to complete dryness so that when you bring it up into a known amount of solution, you will have an accurate idea of the concentration.

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Appendix A. Physical Properties of Common Radionuclides

Radionuclide	Halflife	Beta Energy, max(MeV)	Specific Activity, max
Tritium (hydrogen-3)	12.4 years	0.0186	28.8 Ci/matom[a] 1.06 TBq/matom[a]
Carbon-14	5730 years	0.156	62.4 mCi/matom 2.31 GBq/matom
Sulfur-35	87.4 days	0.167	1.49 kCi/matom 55.3 TBq/matom
Phosphorus-32	14.3 days	1.709	9.13 kCi/matom 338 TBq/matom
Phosphorous-33	25.3 days	0.249	5140 Ci/matom
Iodine-125	59.6 days	Electron capture[b]	2.18 Ci/matom 80.5 TBq/matom[a]

Source: Data reproduced from *Guide to Working Safely with Radiolabelled Compounds* (Amerhsam International, 1974).

[a] A milliatom is the atomic weight of the element in milligrams.

[b] Electron capture is a radioactive transformation in which the nucleus absorbs an electron from an inner orbital. The remaining orbital electrons re-arrange to fill the empty electron shell and in so doing energy is released as electromagnetic radiation at X-ray wavelengths and/or electrons.