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## Introduction

Many biology teachers spend some time on animal development. Generally, training of such teachers has included laboratory experience with the development of amphibian, avian, and echinoderm embryos. Frequently, justification for spending time on development in a variety of courses is that it provides insight into human development. Where this is the case, it seems reasonable to include the development of living mammalian embryos as part of the students' experience.

Advances in mammalian development, over the past 2–3 decades, removed much of the mystery of the young mammal developing in the uterus – at least at the descriptive level. The early development of the mouse (through the hatched blastocyst stage) is very similar to that of humans. This fascinating realm should be made accessible to many undergraduate students.

The procedures described here may be approached at two different levels. Level 1: To provide early mouse embryos of different stages for students to observe living, developing, mammalian embryos. Level 2: To provide cultures of mouse embryos for student observation and, possibly, experimentation.

## Materials

Syringe (1 cc) and saline  
 Paper towels  
 Kimwipes  
 Scissors, 1 (fine, stainless steel, dissection)  
 Forceps, 2 (fine, stainless steel, iridectomy)  
 Bottle of Hoppe medium (100 ml; see Appendix B)  
 Plastic petri dishes, 10 mm × 35 mm, sterile (5)  
 Paraffin oil (in T-25 flask; filled to neck)  
 Empty T-flask (1)  
 Pipets, 1 cc, sterile, individually wrapped (3)  
 Marker (1)

Syringe (1 cc) with blunted needle (30 gauge) (1)  
Mouth tube and mouth piece (1 each per student)  
Transfer pipet (1 per student)  
Desiccator (1)  
Metal probe, for checking for vaginal plugs (1)  
Sharpening stone, for blunting 30-gauge needles (1)  
Diamond-tipped stylus  
Waste oil bottle  
Ziploc bag, quart size (1)  
Slide warmer (e.g., Fisher #12-594)  
Plastic petri dish cover with gas line  
Stereoscopic dissecting microscope with a substage mirror having shiny and dull sides  
Tank of gas (5% CO<sub>2</sub>/95% air) with two-stage regulator  
Screw-top bottles, 100 ml, clean, sterile  
Nalgene filter unit  
Hoppe medium components (see Appendix A)  
Superovulation hormones (see Appendix D)

## Part A: Mice

### Handling Mice

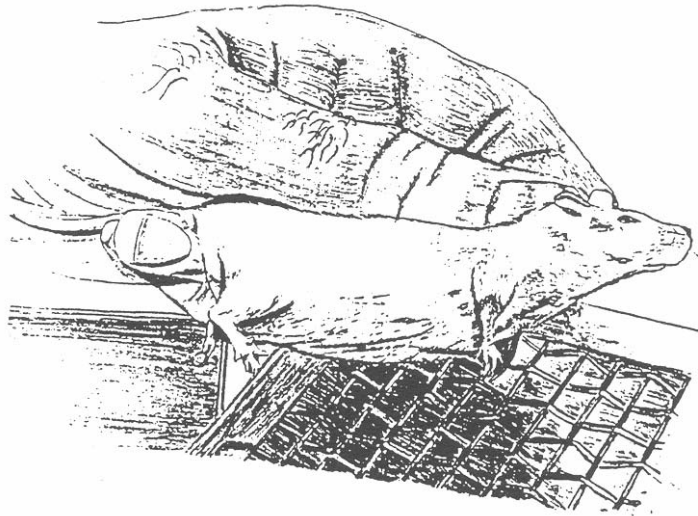
If your experience with mice is minimal, books are available on their biology (Crispens, 1975; Green, 1966), anatomy (Cook, 1965), and histology (Gude et al., 1982).

It is difficult to imagine an animal easier to work with than the laboratory mouse. Mice are small, docile, and economical, and provided with a handle (tail). Nevertheless, unless mice are handled with care, they will be stressed and you may discover how they can use their sharp incisors for defense. Holding a mouse by its tail, for short periods of time, apparently does not cause it to be unduly stressed. However, if you casually hold a mouse by its tail for long periods of time, you will increase the likelihood of being bitten. Sometimes it is necessary to hold the animal for longer than optimum periods of time to perform a given procedure. I have had good experience with the following technique.

Place the mouse on a surface it can grasp, such as a cage top, rather than on a smooth surface over which it can slide. I am describing this technique for right-handed people, if you are left-handed make the appropriate hand changes:

1. Hold the animal by its tail with your right-hand.
2. Bring your left thumb and index finger to the base of the tail and gently push them along the back of the animal to just behind its skull.

3. Next, slide the thumb and index-finger down the sides of the animal's neck to the cage top.
4. Now, reverse direction with your two fingers and as you do so gather up the scruff of the animal's neck between your two fingers .
5. If you now pick up the animal and wrap your last two fingers (ring finger and pinky) around the base of its tail, the animal will be completely secured and, although it would if it could, it cannot bite you (Figure 8.1). After handling the mouse in this manner, *drop* it from a low altitude into a cage bottom containing bedding.



**Figure 8.1.** Holding a mouse for an intraperitoneal (I.P.) injection.

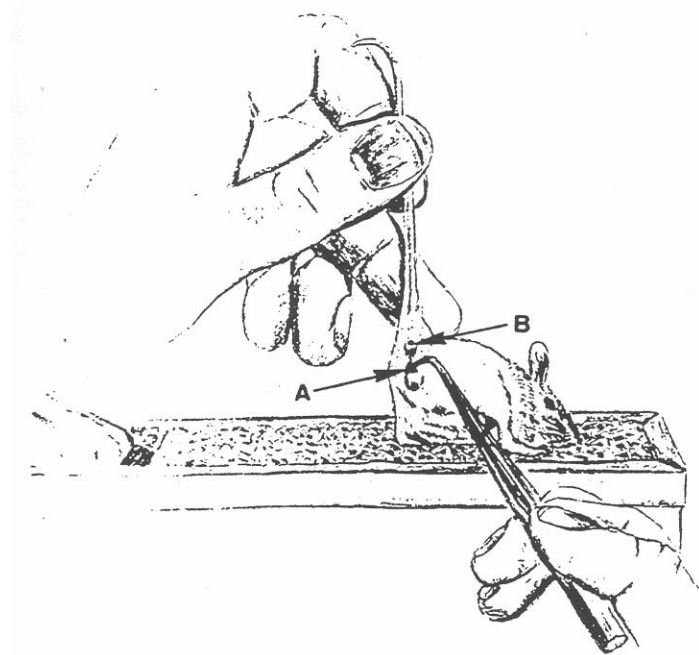
### **Breeding Mice and Aging Embryos**

If you have a minimal background, the books by Rugh (1968) and Theiler (1972) are excellent places to start acquiring a more extensive background in mammalian development.

Mice are easy to obtain, maintain, and breed. Although the number of embryos obtainable from a mouse may be doubled (or more) by induction of superovulation with gonadotropic hormones, and a technique is described below, for the beginner, it is probably wise to leave this complication aside.

I suggest the following timetable for breeding and aging the resulting embryos:

1. Day 1: Place females with the males at 4:00 p.m.
2. Day 2: Check females for vaginal plugs (Figure 8.2), early in the morning (by 10:00 a.m.).
3. Day 2: For zygotes, dissect out the oviducts, locate the ampulla of each oviduct, and prick it with a sharp needle. Zygotes, within a cloud of follicle cells (cumulus), will ooze out of the oviducts.
4. Day 3: For two-cell embryos, flush the oviducts (see Flushing Oviducts in Part B).



**Figure 8.2.** Examining a mouse for a vaginal plug (A, vaginal opening; B, anus)

- Subsequent days: Flush the oviducts/uterine horns for the stage of development desired, up to implantation. Table 8.1 (adapted from Rafferty, 1970) will guide you in choosing the appropriate day for flushing oviducts.

**Table 8.1.** Relationship between time after mating, developmental stage, and location of mouse embryos.

Copulation age	Number of cells	Location
0–24	1	Ampulla
24–38	2	Upper oviduct
38–50	3–4	Mid-lower oviduct
50–64	5–8	Lower oviduct/uterus
60–80	Morula	Uterus
74–82	Blastocyst	Uterus

By following the above schedule, one may obtain a series of developmental stages at 24-hour intervals. Alternatively, one may culture 2-day embryos (see Mouse Embryo Culture in Part C) and obtain successive stages in development.

## Vaginal Plugs

The male mouse has coagulating glands, secretions of which leave a mucoid plug in the vaginal opening. Frequently, this vaginal plug is quite obvious. If not, gentle probing of the vagina with a metal probe will often reveal the presence of a plug (Figure 8.2). If a vaginal plug is found, you are reasonably sure that conception has occurred and can handle the mouse according to the above "Aging Embryos" timetable (Table 8.1). Good background references for Parts B and C, below, are the books by Daniel (1971), Hogan et al. (1986), and Rafferty (1970).

## Part B: Obtaining Embryos

### Superovulation

The yield of eggs or embryos obtainable from a single mouse may be increased by superovulation. The references by Rafferty (1970) and by Daniel (1971) should be consulted for background information. In my experience, the following procedure has worked well.

Although other sources should be efficacious, the two hormones used, pregnant mare's serum (PMS) and human chorionic gonadotropin (hCG), may be obtained from Sigma Chemical Co. Each hormone is dissolved in sufficient 0.9% NaCl to yield a final concentration of 25 i.u./ml. See Appendix D.

Female mice are injected, at 4:00 p.m., intraperitoneally (I.P.) with 0.2 ml of PMS (= 5 i.u.). Forty-eight hours later, the mice are injected, I.P., with 0.2 ml of hCG (= 5 i.u.). Immediately after the second injection, injected females are placed with males. Early the following morning, female mice are examined for vaginal plugs.

In my experience, as many as 40 two-cell embryos have been obtained from a single female with this regimen. However, I have found the yield to vary enormously, even among females that are litter mates and injected simultaneously. The above references, and perhaps the original literature, should be consulted before spending much time with this procedure.

### Isolation of Oviducts (aseptic procedure)

Isolation of the oviducts is an obvious prerequisite to flushing them. Sacrifice the animal by cervical dislocation, as follows (for right-handed people):

1. Place the animal on a rough surface, so it does not slide about.
2. Hold the animal by the tail with your right-hand.
3. Bring your left thumb and index-finger to the base of the tail and gently push them along the back of the animal to just behind its skull. Stabilize the position of the skull with these two fingers.
4. With your right-hand, give a smooth, firm, backward jerk of the tail.
5. If done competently, the animal should be dead humanely and instantaneously.

Dissection of the animal should be done swiftly and cleanly:

1. Position the animal on its back and swab the abdominal skin with 70% ethanol.
2. Lift the skin away from the underlying musculature and make a short (1–2 cm) incision through the abdominal skin, at right-angles to the longitudinal axis of the animal.
3. Grasping the skin on both sides of the incision, pull your hands apart, peeling the skin back from the underlying musculature (Figure 8.3).
4. Open the abdominal (peritoneal) cavity by your standard procedure.
5. Gently, push the intestines out of the abdominal cavity.

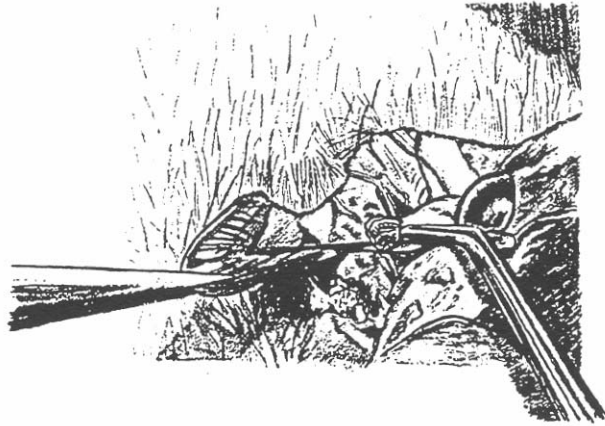


**Figure 8.3.** Reflecting the skin to expose the wall of the peritoneal cavity.

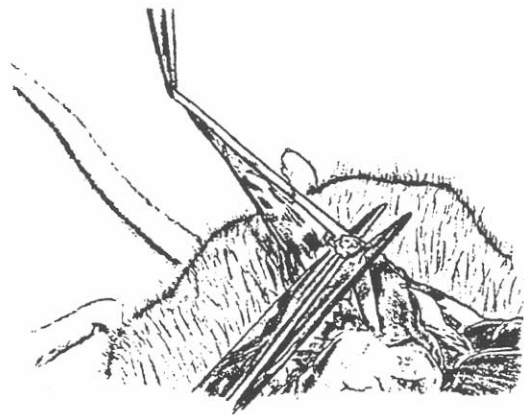
In the posterior-half of the abdominal cavity, locate the two uterine horns and the short body of the uterus; these three structures have an inverted “wishbone” configuration. If you gently pull, with forceps, on one of the uterine horns, you will find, at its anterior end, the tiny oviduct between the end of the uterine horn and the tiny ovary (Figure 8.4).

While exerting slight tension on the uterine horn, in a direction opposite to that of the ovary, with fine scissors make a cut between the oviduct and the ovary. Then, make a second cut across the uterine horn just behind your forceps (Figure 8.5).

With the forceps, transfer the short length of horn and attached oviduct to a small petri dish containing Hanks balanced salt solution (HBSS; Grand Island Biological Co., GIBCO) or another mammalian physiological saline, for observational purposes. If you plan to culture the embryos, substitute Hoppe medium for the saline. In either case, the embryos will do better if they are kept in an atmosphere of 5% carbon dioxide and 95% air on a warm surface (e.g., a slide warmer) at 37°C.



**Figure 8.4.** Locating the oviduct. The forceps (*left*) is pulling on a uterine horn; the probe (*right*) is pointing to the oviduct at the end of the horn.

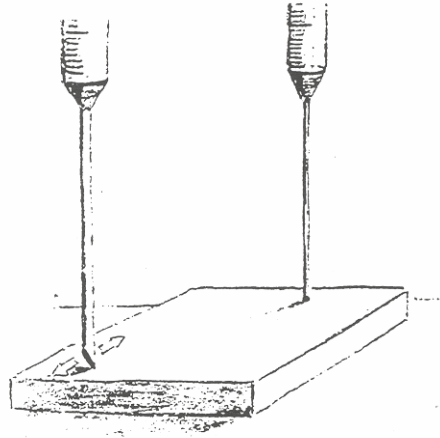


**Figure 8.5.** As forceps pull on a uterine horn, scissors cut between the oviduct and the ovary.

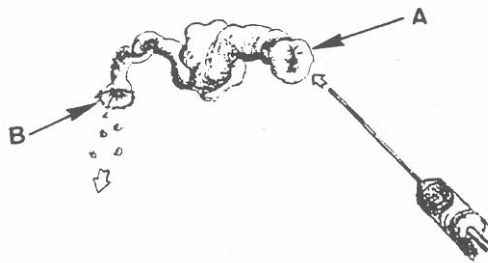
### **Flushing Oviducts (aseptic procedure)**

This next part of the procedure requires a syringe needle, suitably prepared, and a dissecting microscope. Blunt the tip of a 30 gauge needle by rubbing it across the surface of a sharpening stone (Figure 8.6); this will prevent the needle from piercing the wall of the oviduct once you have inserted it through the opening (ostium) at the end (ampulla) of the oviduct.

Fill a 1-cc syringe with physiological saline and attach the blunted needle to it. Place the dish of saline containing the oviduct, on the glass stage of a stereoscopic, dissecting microscope and locate the oviduct with bright transmitted light. The ampulla of the oviduct is slightly dilated and has the ostium at its end (Figure 8.7).



**Figure 8.6.** Blunting (removing the bevel) a 30-gauge needle by rubbing its point back and forth across a sharpening stone.

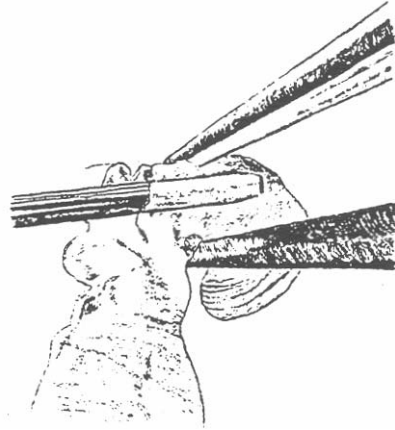


**Figure 8.7.** What flushing the oviduct is to accomplish: injecting fluid into the ampulla and flushing embryos out the end of the segment of uterine horn (*A*, ampulla of the oviduct with needle approaching ostium; *B*, cut end of the uterine horn).

Hold a fine, stainless steel forceps (iridectomy) in your left hand and the 1-cc syringe (with attached, blunted, 30-gauge needle) in your right hand. With these two instruments, search through the coils of the oviduct to locate the ampulla and ostium. Once located, grasp the ampulla, ever so gently, with the forceps and thread the end of the needle into it (Figure 8.8).

Once the needle is in the ampulla, use the forceps to clamp the wall of the oviduct around the end of the needle and, then, gently and smoothly, press on the plunger of the syringe. As the saline is pushed through the oviduct, the oviduct will “blow up” like a child's long, party-balloon being inflated and the contained embryos will be flushed out of the cut end of the uterine horn (Figure 8.7).

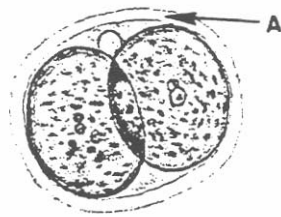
If you plan to simply use the embryos for short-term observational purposes, use a physiological saline in the syringe, as described above. However, if you intend to culture the embryos, substitute Hoppe medium for the saline.



**Figure 8.8.** The blunted needle inserted into the ampulla, with the forceps just about to clamp the wall of the oviduct against the needle.

**Collecting Embryos** (aseptic procedure)

Remove the oviduct from the saline (or Hoppe) medium and discard it. Turn the substage mirror of the dissecting microscope so that its dull side is now uppermost and, using the highest magnification of your microscope, search the floor of your petri dish for embryos. Early embryos are surrounded by the hyaline (clear), noncellular, zona pellucida which is an excellent embryo marker for your search (Figure 8.9). The dish of embryos may be placed on the stage of a microscope for immediate student observation or embryos may be placed in embryo culture for immediate and successive observations (see Part C).



**Figure 8.9.** A two-cell embryo (A, zona pellucida).

## Part C: Mouse Embryos

### Handling Embryos (aseptic procedure)

An inexpensive way to handle mouse embryos is to use embryo transfer pipets, pulled from clean, sterile pasteur pipets. It is necessary to pull both ends of the pasteur pipet. The narrower end is pulled to a fine inner diameter (about 125  $\mu\text{m}$ ) to accommodate the embryos and medium. The wide end of the pasteur pipet is pulled to accommodate the inner diameter of the mouth tube that you are using. Naturally, both ends of the pipet are pulled after being sufficiently heated in the flame of a bunsen burner. Care should be taken to avoid burning your fingers. A diamond tip stylus may be used to score and break both ends of the pipet at the proper diameters.

The “mouth end” of the embryo transfer pipet is inserted into the end of a length of rubber tubing to which a mouthpiece is attached (e.g., Fisher Scientific Co. #13-647-5). The embryos are gathered into and expelled from the transfer pipet by mouth control. An important point is to dip the end of the transfer pipet into the medium you are using and let the liquid rise in the pipet by capillary action; then you may safely approach embryos with the tip of the transfer pipet. Also, once the transfer pipet is loaded with medium in this fashion, you can pass the pipet tip through the paraffin oil overlying the drops of culture medium (see Mouse Embryo Culture, below) without pulling oil into the tip of your transfer pipet.

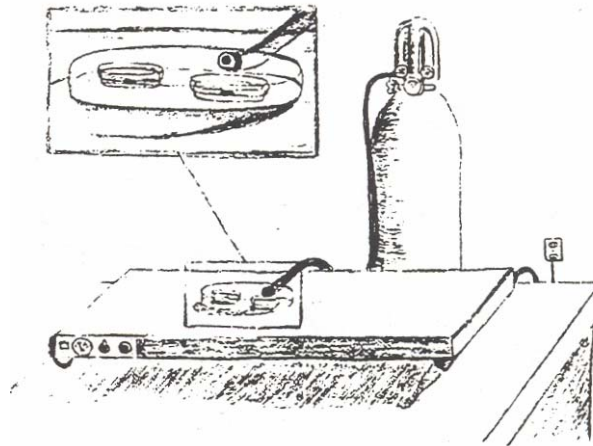
### Mouse Embryo Culture (aseptic procedure)

Background reading for mouse embryo culture should include the papers by Brinster (1972) and Sherman (1979). A convenient culture vessel for mouse embryos is a small (10  $\times$  35 mm), sterile, disposable, plastic petri dish. Microdrops of medium are placed on the floor of the dish with a sterile 1-ml pipet or with a sterile embryo transfer pipet. One 0.5-cm diameter drop will accommodate several embryos or as many as 25 microdrops of medium may be placed on the floor of a dish with an embryo transfer pipet; this is especially useful if you want to keep embryos in separate drops of medium to keep track of specific embryos.

The drops of medium are covered by a layer of sterile paraffin oil (see Appendix C), which is added by simply pouring it gently into the bottom of the petri dish until each drop of medium is completely covered. The oil will prevent evaporation from the small drops of medium.

When dishes of medium are not being manipulated on the stage of the microscope, they should be exposed to an atmosphere of 5% carbon dioxide in air. This is conveniently done by having a tube convey the gas from its cylinder, controlled by a two-stage regulator, to a large, transparent, plastic petri dish lid (e.g., the top of a large, 20 cm, petri dish) which is placed over the small petri dishes of embryos when they are not being handled. Some people also keep the embryo cultures on a slide warmer kept at 37°C when the cultures are out of the incubator for prolonged periods of time (Figure 8.10).

If a carbon dioxide incubator is not available, an ordinary glass chemical desiccator may be adapted for this purpose. Water is placed in the bottom of the desiccator, beneath the porcelain plate. The ground glass joint between the desiccator bottom and lid should be adequately greased to provide a gas-tight seal and the desiccator should be sterilized by autoclaving with the lid slightly open. Culture dishes are placed on top of the porcelain plate. The desiccator is flushed out with 5% carbon dioxide in air, which may be delivered to the desiccator by means of a rubber tube and 10 ml pipet. If the gassed desiccator is now placed in an incubator, you will have a “poor man's carbon dioxide incubator.”



**Figure 8.10.** Embryo-containing dishes on a slide warmer, under a large petri dish lid which is being gassed.

### Acknowledgements

I greatly appreciate the work of my illustrator, John C. Dye, who created the figures in this chapter. I also wish to recognize those who introduced me to “hands on” early mammalian embryology: Professor Clement Markert and Drs. Mike Bradbury, Bruce Pratt and Mary Rose Klein, all, then, of the Biology Department at Yale University.

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APPENDIX A  
*Source of Chemicals for Hoppe Medium*

Listed below are the organic chemicals I use and their commercial sources (the other components of the medium, glucose and salts, are from grades of highest purity):

1. Pentax Bovine Albumin, crystallized (#81-001-3): Miles Laboratories, Inc., Research Products Division, Naperville, Il 60566.
2. L(+) Lactic Acid (Hemi-calcium salt, hydrate grade L-IV): Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.
3. Sodium Lactate, 60% (#11773): Pfanstiehl Laboratories, Inc., 1219 Glen Rock Ave., Waukegan, IL 60085.
4. Pyruvic Acid (Na salt, Type II, crystalline; #P-2256): Sigma Chemical Co.
5. Penicillin "G" Sodium (#180030): Grand Island Biological Co. (GIBCO), Grand Island, NY 14072.
6. Streptomycin Sulfate (#180060): GIBCO.
7. Phenol Red Solution, 0.5% (#510): GIBCO.

APPENDIX B  
*Culture Medium*

The formulation for the medium given below is a modification of Hoppe Medium as used in the laboratory of Professor Clement Markert at Yale University.

Bovine serum albumin	0.3000 g
NaCl	0.4700 g
KCl	0.0356 g
KH <sub>2</sub> PO <sub>4</sub>	0.0162 g
MgSO <sub>4</sub> (7H <sub>2</sub> O)	0.0294 g
NaHCO <sub>3</sub>	0.1900 g
Glucose	0.1000 g
Calcium lactate (5H <sub>2</sub> O)	0.0527 g
Sodium lactate syrup (60%)	0.3680 ml
Sodium pyruvate	0.0025 g
Penicillin G	0.0075 g
Streptomycin sulfate	0.0050 g
Phenol red, 0.5%	0.2000 ml
2X distilled water	up to 100 ml

In preparing the medium, I suggest that you use two 100 ml, tissue culture clean, sterile, screw-top bottles. Place about 90 ml of water (distilled in glass twice or of comparable quality) in one of the bottles. Except for the albumin, add the other 10 dry ingredients successively to the water; dissolve each of these before adding the next substance. Next, add the two liquid ingredients. Before the next addition, see the note at the end of this appendix! Then, carefully sprinkle the albumin onto the top of the solution and allow it to dissolve without agitation; this will take a few minutes. Finally, add water up to the 100 ml level.

The medium should now be sterilized by filtration. A handy method is to use a Nalgene filter unit, with a pore size of 0.22  $\mu$ m. Do not pull the medium through the filter with a vacuum as this will cause excessive frothing. Either push the medium through the filter with compressed gas (5% carbon dioxide in air) or allow it to flow through the filter by gravity.

Aseptically transfer the medium to the second bottle. Label the bottle of sterile medium, including date of preparation. The medium, kept refrigerated, will be good for about 2 weeks.

*Note:* If you are going to equilibrate paraffin oil (see Appendix C) with medium, you need to set aside 10 ml of medium without albumin before you add the albumin to the rest of the medium. Therefore, bring the medium up to 100 ml with water, set aside 10 ml of the medium for equilibration, and add 0.2700 g of albumin instead of 0.3000 g, as described above, to the remaining 90 ml of medium.

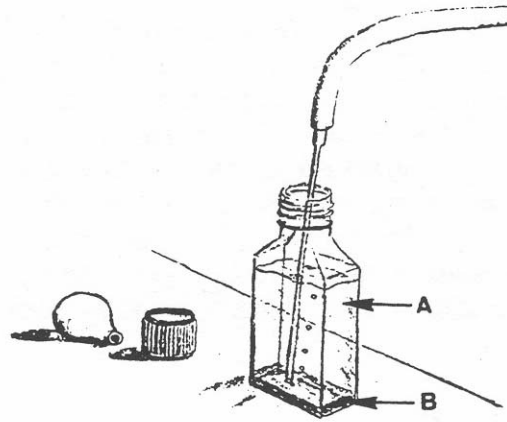
APPENDIX C  
*Equilibration of Paraffin Oil*

The paraffin oil, which overlies the microdrops of medium, is equilibrated with two ingredients: 5% carbon dioxide gas and growth medium. If this is not done, the oil will leach components out of the growth medium which will alter the medium's composition.

Paraffin oil may be equilibrated with both the gas and the growth medium simultaneously. Add 10 ml of the sterile medium (minus the albumin) to a sterile, disposable, Falcon T-25 flask, then add sterile oil up to the neck of the T-flask. Not all investigators believe it is necessary to sterilize the paraffin oil.

Pull a sterile pasteur pipet out to a fine tip and, then, attach the mouth end of the pipet to a tube coming from a cylinder of 5% carbon dioxide in air. Insert the pulled end of the pipet into the medium at the bottom of the T-flask and bubble the gas through the medium as vigorously as possible, consistent with the oil not overflowing the mouth of the flask. Obviously, throughout this procedure, the T-flask is held with its neck up (i.e., not in its normal position when used as a cell culture vessel, Figure 8.11). After a few minutes the color (amber) of the medium, due to its contained phenol red, should indicate the appropriate pH. At this point, cap the T-flask with its screw-top cap and shake the flask vigorously.

Put the emulsified medium aside until the oil and aqueous phases separate (overnight). The oil may then be used to overlie the drops of growth medium and it should not leach out components of the medium.



**Figure 8.11.** Equilibrating paraffin oil with gas and medium (A, paraffin oil; B, medium).

APPENDIX D  
*Superovulation Hormones*

The following are suggested dilutions of the commercial preparations which I have found to be convenient:

**hCG (Sigma #CG-10)**

1. Chorionic gonadotropin, 10,000 i.u.
2. Reconstituted with 10 ml saline (0.9% NaCl in sterile, glass distilled water) = a stock solution of 1000 i.u./ml.
3. 1 ml of stock solution brought up to 40 ml with saline = a working solution of 25 i.u./ml, for injection.

**PMS (Sigma #G-4877)**

1. Pregnant mare's serum, 1000 i.u.
2. Reconstituted with 2.5 ml saline = a stock solution of 400 i.u./ml.
3. 1 ml of stock solution brought up to 16 ml with saline = a working solution of 25 i.u./ml, for injection.