

GLUCOSE CLAMPING THE CONSCIOUS MOUSE: A LABORATORY COURSE



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Preface

The development of methodology to manipulate the germline of the mouse has resulted in a dramatic increase in mouse models of disease. This, in turn, has spawned a need to develop technology to study the mouse by overcoming barriers related to their limited size. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) initiated the Mouse Metabolic Phenotyping Center (MMPC) Program in 2001 to address this need. Vanderbilt University School of Medicine has been an MMPC (www.mmpc.org) site since the inception of the program. The objective of the MMPC Program is to develop and apply techniques for studying mouse models of diabetes and related disorders.

“Glucose Clamping the Conscious Mouse: A Laboratory Course” was developed and first conducted in 2005 by the Vanderbilt MMPC based on two needs of the scientific community. The first is the vast number of mice whose initial phenotypes indicate an insulin clamp would be informative in understanding the effect of a transgene or mutation. The second is that until very recently there has been no description or critical discussion of the insulin clamp technique in the scientific literature. The result of this is that there is little convention amongst researchers using this technique. This has resulted in an inability to seriously interpret clamp data and compare clamp data between different laboratories. This course is intended to (1) aid those laboratories needing to perform insulin clamps on a regular basis in implementing the usage of this technically challenging technique at their institution; (2) increase the transparency of the technology so that the factors involved in insulin clamping the mouse is better understood by scientists wishing to make sense of the growing literature; and (3) serve as a forum for scientists interested in study of the mouse *in vivo* to compare and exchange methodology.

As we hope to impart our experiences in glucose clamping the mouse to the participants of this course, we also hope to learn from the participants as they acquire experience. We fully expect for people undertaking this technique to add to and improve upon procedures over time. Our hope is that participants will share their experiences after they return home so that we may all work together to expedite advancements in understanding, preventing, and treating diabetes and other metabolic diseases.

The pages that follow describe technical aspects of the surgical, experimental, and analytical procedures necessary to perform the glucose clamp in the conscious mouse. Dr. Masakazu Shiota, of our faculty, developed the key surgical and experimental technology. The technical description of the procedures was provided by Tasneem Ansari, Bingle Bracy, Freyja James, Lianli Ma, Merrygay James, Teri Stevenson, Alicia Lorance and Carlo Malabanan, all of whom are experts in the many facets of taking a mouse from surgery through to an insulin clamp. David Wasserman, who is not, compiled the manual. Errors and ambiguities are his fault. Please make us aware of sections that need to be clarified or errors that may exist.

The course also benefits from the expertise of Dr. Fu-Yu Chueh and Dr. Li Kang. Last but not least, we sleep much better at night knowing that MMPC administrator Fran Tripp has the logistics of this week covered.

Course Directors,



David Wasserman



Owen McGuinness



Julio Ayala

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1

Introduction

A *glucose clamp* is where arterial glucose is maintained at or near a pre-defined set point by a variable infusion of glucose. The rate of the variable glucose infusion is determined by feedback obtained by frequent measurement of blood glucose. There are several terms that are used, sometimes interchangeably, when referring to the glucose clamp. Since many terms are used, even within the same institution, some expressions should be defined at the onset.

The most common application of the glucose clamp technique is where insulin is infused to create hyperinsulinemia, while arterial glucose is clamped at fasting levels. This is a *hyperinsulinemic, euglycemic clamp*. Since this is a cumbersome expression, it is often referred to simply as an *insulin clamp*. The term, *insulin clamp*, is used in this manual and will be used during the Course. Arterial glucose can also be clamped at a low concentration during a high insulin infusion to test the glucose counterregulatory system, or the ability of the body to respond to hypoglycemia. This is a *hypoglycemic clamp*. Arterial glucose can also be clamped at elevated concentrations without the infusion of insulin. This is called a *hyperglycemic clamp*, and is used to test the secretory capacity of pancreatic β -cells.

It is valuable to be aware of the various applications of the glucose clamp technique. The primary focus here will be on the *insulin clamp*. Once the tools necessary to conduct an insulin clamp are in hand, other clamp protocols can be easily implemented.

Glucose clamping of the conscious mouse requires three fundamental steps. These are 1) the surgical implantation of catheters; 2) postoperative care of the mouse and catheter maintenance; and 3) the clamp experiment. It is important to recognize that there is more than one way to clamp a mouse. While the insulin clamp as performed at the Vanderbilt-NIH MMPC is taught here, there are likely to be other approaches that may also accommodate one's needs. As such, there are many factors that must be considered in designing and interpreting insulin clamp experiments. The hope is that participants will leave here with not only the technical skills necessary to glucose clamp the conscious mouse, but also to better understand how to interpret the results that glucose clamps yield.

2

Surgical Catheterization of the Carotid Artery and Jugular Vein

Study of the unstressed mouse requires prior implantation of catheters in the carotid artery and jugular vein. The arterial catheter is used to obtain samples during the clamp and the venous catheter is used as an infusion port. The procedure used by the MMPC was developed by Dr. Masakazu Shiota of Vanderbilt in the mid 1990's. The surgical procedure is performed under sterile conditions and takes an experienced surgeon <1 h. It is critical not only that the catheters work the day of the experiment, but also that mice are stress-free and infection-free.

Surgical Catheterization of the Carotid Artery and Jugular Vein

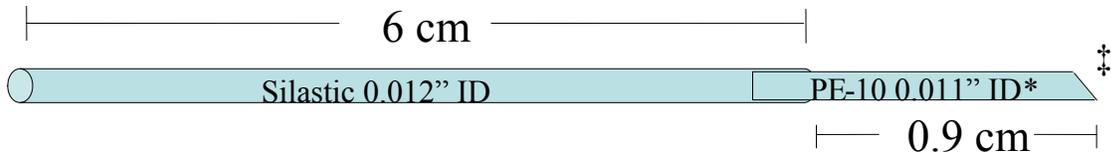
1. Catheter and MASA_m Construction

Supplies:

Silastic tubing 0.012" ID	Abrasive stone medium grain
Silastic tubing 0.020" ID	Stainless steel tubing 25 gauge 0.0123" ID
Silastic tubing 0.058" ID	Stainless steel wire – 0.02" OD
PE-10 0.011" ID	Graefe forceps
PE-20 0.015" ID	Silicone medical adhesive
File	Dumont 7b curved shank forceps

Arterial Catheter Preparation (Figure 1):

1. Cut 10 cm of PE-10 and carefully stretch the tubing to ~13 cm. If the tubing is stretched much beyond this point it will snap.
2. Cut stretched PE-10 into 1.3 cm pieces.
3. Cut silastic tubing (0.012"ID) into 6 cm pieces.
4. Insert Dumont forceps into the end of a 6 cm piece of silastic. Gently open forceps so the tubing is opened wide.
5. Gently grasp the end of a stretched and pre-cut piece of PE-10 using a pair of Graefe forceps, and insert the tip of the PE-10 into the silastic tubing approximately 3 mm.
6. Slip the Dumont forceps from the silastic tubing, leaving the PE-10 in place. The catheter tip is beveled at the time of surgery.



*Catheter is stretched.

‡Bevel tip with scalpel

Figure 1. Carotid Artery Catheter. Scheme is not to scale. Note also that exact dimensions may vary depending on the size of the mouse.

Venous Catheter Preparation (Figure 2):

1. Cut a 6 cm piece of silastic tubing (0.012" ID).
2. Cut a 1 mm section of silastic tubing (0.020" ID) for use as restraining bead.
3. Insert the tips of eye dressing forceps into the lumen of the restraining bead and gently hold the tips of the forceps apart to stretch the opening wider.
4. Using another pair of eye dressing forceps, slide the silastic tubing into the lumen of the restraining bead.
5. Pull the silastic tubing through the bead until it reaches 11 mm.
6. The bead must lie flat around the catheter.
7. Adjust the bead and the bevel catheter tip at the time of surgery making any necessary adjustments for differences in mouse size.

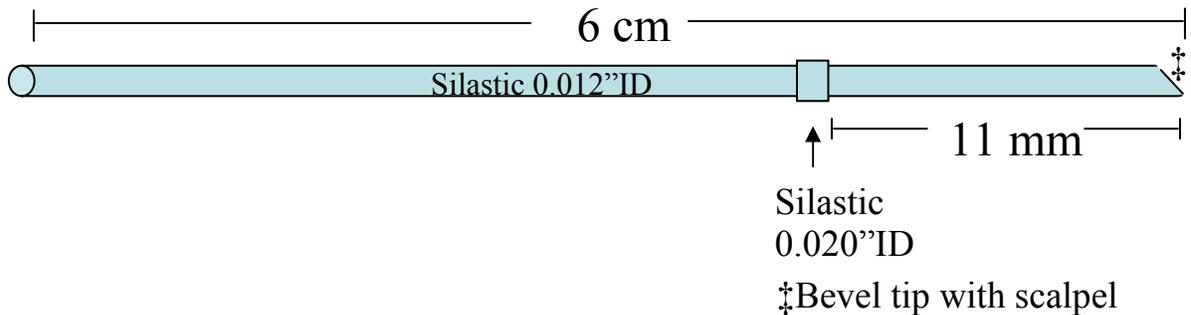


Figure 2. Jugular Vein Catheter. Scheme is not to scale. Note also that exact dimensions may vary depending on the size of the mouse.

MASA_{tm} Preparation (Figure 3):

1. Cut two 13mm stainless steel tubes using file, smoothing edges with abrasive stone.
2. Bend each stainless steel tube in half at a 120° angle.
3. Attach PE-20 to one end of each bent tube.
4. Slide silastic tubing (0.058" ID) over where both steel tubes and PE-20 is attached.
5. Place completed rig in silicone medical adhesive for 24 hours.
6. Catheter plugs for MASA_{tm} are cut from steel wire for use at time of surgery.

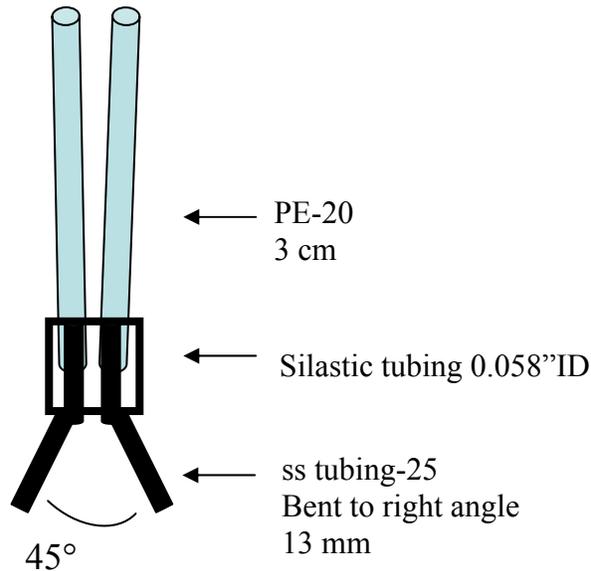


Figure 3. MASA_{tm}. Scheme is not precisely to scale.

2. Surgical Procedures

Supplies:

Pentobarbital	200 U/ml heparinized saline
Arterial catheter (Figure 1)	Plugs
Venous catheter (Figure 2)	Small gauge syringe
MASA _{tm} (Figure 3)	Ruler
Graefe forceps	Cautery
Surgical scissors	Scalpel
Betadine scrub solution	Saline
Spring scissors	70% alcohol
Needle holder	7-0 nylon suture
Micro-serrefines	6-0 silk suture
14-gauge needle, dulled	

**All surgical equipment must be sterilized.*

Preparation:

1. Anesthetize mouse (40 - 85 mg/kg pentobarbital ip or with isoflurane). Bevel catheters to correct lengths, fill with heparinized saline and plug.
2. Surgery must be conducted in a disinfected area that promotes asepsis.
3. Prepare the animal by removing hair from the surgical site. Perform this procedure in an area separate from where the surgery is to be conducted.
4. Prepare the surgical sites with an appropriate skin disinfectant (betadine followed by alcohol scrub).

5. Surgeons wash and dry their hands before aseptically donning sterile surgical gloves.

Surgery:

1. Make small vertical midline incision 5 mm cephalic to the sternum.
2. Blunt dissect using forceps to expose the left sternomastoid muscle. Reflect this muscle to expose left carotid artery.
3. Gently tease off connective tissue from the carotid artery. It is important at this point to isolate the vagus nerve from the artery without damaging either the artery or the nerve. Isolate artery then ligate cephalic end with silk suture. Another piece of suture is loosely knotted on the caudal end of the exposed vessel.
4. Clamp vessel with micro-serrefine and cut just below the ligated end with spring scissors. Carefully insert catheter as far as possible. Grab catheter with forceps, then carefully release micro-serrefine clamp and continue inserting catheter to the silastic-polyethylene junction. At this point the tip of the catheter should be in the aortic arch.
5. Tie both ligatures securely and confirm that the catheter samples. Flush with heparinized saline.
6. Make another incision 5 mm to the right of midline and about 2 mm caudal to the first incision.
7. Blunt dissect to expose right jugular vein.
8. Isolate carefully and ligate cephalic end with silk suture. Loosely tie another piece of suture at the caudal end of the exposed vein.
9. Cut just below cephalic ligature with spring scissors. Insert catheter to the bead, tie and confirm that it samples. Flush with heparinized saline.
10. Turn mouse over. Make a small incision between shoulder blades. Tunnel 14-gauge needle under skin through the incision on the back. Thread catheters through the needle to exteriorize them at the back of the mouse.
11. Close ventral incisions with nylon suture.
12. Clamp venous catheter with micro-serrefine at the incision site between shoulder blades. Cut catheter 1 cm above clamp and connect to MASA_{tm}. Take care to ensure that there are no holes or kinks in the catheter. Secure venous catheter to MASA_{tm} with silk suture. Repeat for the arterial catheter.
13. Close dorsal incision with nylon and confirm patency of both catheters again. Flush with heparinized saline. Place mouse in warmed, clean cage.

3

Postoperative Care and Catheter Maintenance

Proper attention to the well-being of the mouse and catheter patency during the interval between surgery and experiment determines, to a large extent, the quality of the results obtained using an insulin clamp. Described in this section are the tools and reagents needed and procedures used to sustain the integrity of the chronically catheterized mouse model during the critical postoperative period. The text below describes some observational tools to assess pain, distress or infection. Consult your institutional veterinarian if adverse symptoms appear or concerns over the condition of the mouse otherwise arise.

Postoperative Care and Catheter Maintenance

Supplies:

Clean cage (fresh bedding, food, fresh water bottle)
Cage heater pad
TB syringe – 1 ml (without needle),
Blunt needle with luer hub (25 G x 1/2")
Microrenathane tubing
Connector
Flush Solution (200 U/ml heparin in saline and 5 mg/ml ampicillin)
Regular Clamps
Regular Clamps with silicon tubing over tips.
Streptokinase (Streptase; 25,000 Units/ml)
Cannulation System (8 cm)

1. Postoperative Care of the Mouse

Preparation (as mouse goes into surgery):

1. Put clean cage on heating pad.
2. Check to ensure that the water bottle does not leak and contains fresh water. Appropriate food should be placed in the cage. Place some food on cage bottom.

Immediate Postoperative Care (as mouse comes off table):

1. Check the water bottle for leaks, then place animal belly down on warm cage bottom. Watch for excessive bleeding from incisions, the mouse catching its catheter lines in the cage lid, or the animal scratching its sutures open.
2. After animal is fully ambulatory and aware, remove cage from heating pad to the housing area.

Daily Postoperative Checklist:

1. *Observe animal for signs of infection.* Infection is indicated by suppuration from incision sites, general lethargy and/or pain.
2. *Watch mouse for signs of pain.* Most healthy animals are ambulatory and eating within 2 h after the completion of this procedure. Pain may be indicated by a hunched posture and ruffled fur. The mouse may also consume bedding instead of food if in pain. Follow recommendations of veterinarian advice as to how to alleviate pain and infection.

3. *Check animal for signs of stroke.* The animal will twist abnormally when lifted by the tail, and it may also show limb weakness and difficulty walking. It may have difficulty righting itself in response to a gentle push. Although an animal may recover occasionally, most do not. Euthanasia is suggested.
4. *Weigh mouse.* The mouse may lose weight immediately following surgery, but by the third day the weight loss should stop. By the fifth day, the animal should be within 10% of its original weight. Excessive weight loss may indicate infection, discomfort, and/or stroke.

2. Maintaining the Catheter Lines

1. Fill syringe with Flush Solution and cap it with luer hub. Luer hub is inserted into microrenathane tubing with connector at the other end (see Figure 4).

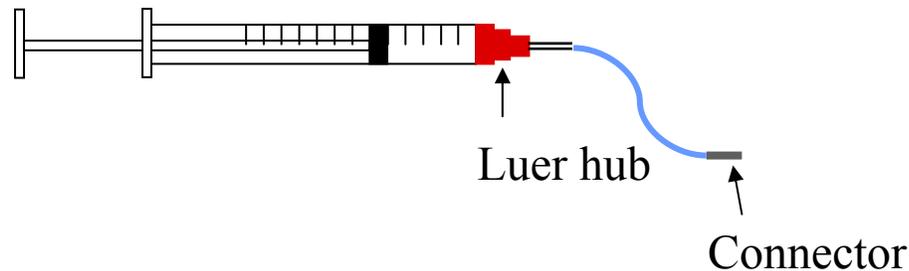


Figure 4. Flushing syringe. Blunted tip of Luer hub is inserted to the microrenathane lumen.

2. Remove air bubbles by placing the end connector higher than the rest of the flush syringe and pushing the bubbles out of the line.
3. Allow the flush solution in the syringe to warm up before actually putting it into the animal. (Hold it between your hands until it feels warm.)
4. Place the mouse on the cage top.
5. Clamp off the arterial line (on the mouse's left side) with silicone tipped forceps just below the plug.
6. Remove the steel catheter plug using the second pair of forceps.
7. Slip the connector end of the flush line into the arterial line.
8. Release the silicone tipped forceps.
9. If the catheter tip is positioned correctly in the aortic arch then blood can be aspirated easily. Aspirate content of the catheter into syringe. If the catheter does not draw easily it may be necessary to very gently push in a small amount of flush

solution through the catheter to dislodge the tip of the catheter in case it is wedged against the vessel wall*. The catheter has been completely cleared when a plume of blood reaches the syringe. Do not withdraw any more blood than is absolutely necessary to clear the catheters, as blood volume is limiting on the day of the experiment.

10. Clamp the microrenathane tube close to the syringe and dispose of syringe.
11. Replace with a new syringe filled with fresh and warmed flush solution.
12. Unclamp the microrenathane tubing and holding the new syringe upright, flick the new syringe with a finger nail to dislodge any possible new air bubbles upwards and away from the line. GREAT CARE MUST BE TAKEN TO PREVENT INJECTION OF AIR BUBBLES, AS STROKE CAN RESULT.
13. Inject flush solution until the catheter line is clear and blood-free. Push in an additional 0.04 ml to ensure mouse receives adequate amounts of ampicillin.
14. Clamp the arterial catheter line with the silicone tipped forceps below the connector of the flush syringe. Slip the syringe connector from the catheter line and replace it with the steel plugs. Remove the silicone tipped forceps from the arterial line.
15. The venous line (the one on the mouse's right side) is cleared in a manner similar to the arterial line. However, because of the low venous pressure sampling is often not possible. If flush solution can be infused with minimal resistance it is probable that the catheter is well positioned in the vein.

* In the case of severe clotting, the catheter can be filled with streptokinase to dissolve the clot as described in the Streptase package insert. The wire from the cannulation system can be inserted into the catheter to the base of the bend of the wire connectors of the MASA_{tm} to further dislodge clots. Be aware than any clot will cause a problem if it is pushed into the circulation. Clots must be aspirated into the discard syringe and disposed with it.

Confirm the position of both catheters at the time of necropsy, which will hopefully be at the end of the experiment.

4

The Insulin Clamp

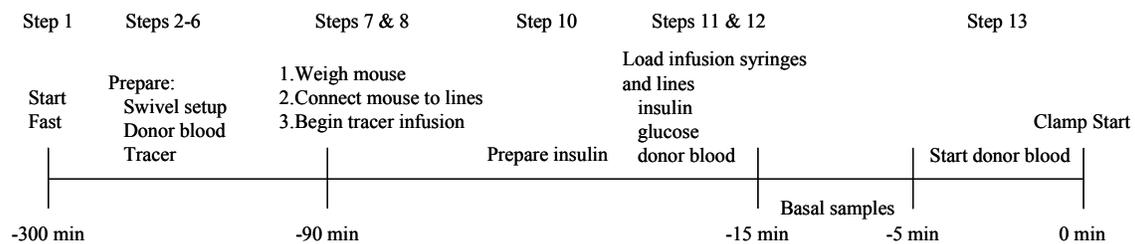
The hyperinsulinemic, euglycemic clamp or “insulin clamp” has been used in a variety of species to assess insulin action. In an insulin clamp, the rate of glucose infused to maintain euglycemia is an index of whole body insulin sensitivity. Isotopes can be used during an insulin clamp to distinguish between insulin’s effects on endogenous glucose production and glucose utilization, or to examine insulin’s effects on specific tissues and metabolic pathways. The insulin infusion rate used in a study depends on a number of factors such as whether the experiment requires a sensitive analysis of endogenous glucose production, or whether the mouse is known to be unusually insulin resistant (e.g. ob/ob). Finally the insulin clamp technique, especially when isotopes are involved, is most quantitative under steady state conditions. Therefore, clamps should be of a duration (~2 h) that is sufficient for the actions of insulin to obtain steady state. The section that follows describes, a) the technical procedures used on the day of a clamp experiment; b) preparation of insulin and tracer infusates; and c) analyses of plasma and tissue radioactivity. Familiarity with these procedures combined with laboratory demonstration provides the investigator with the necessary background to clamp the conscious mouse.

The Insulin Clamp

Equipment and Supplies:

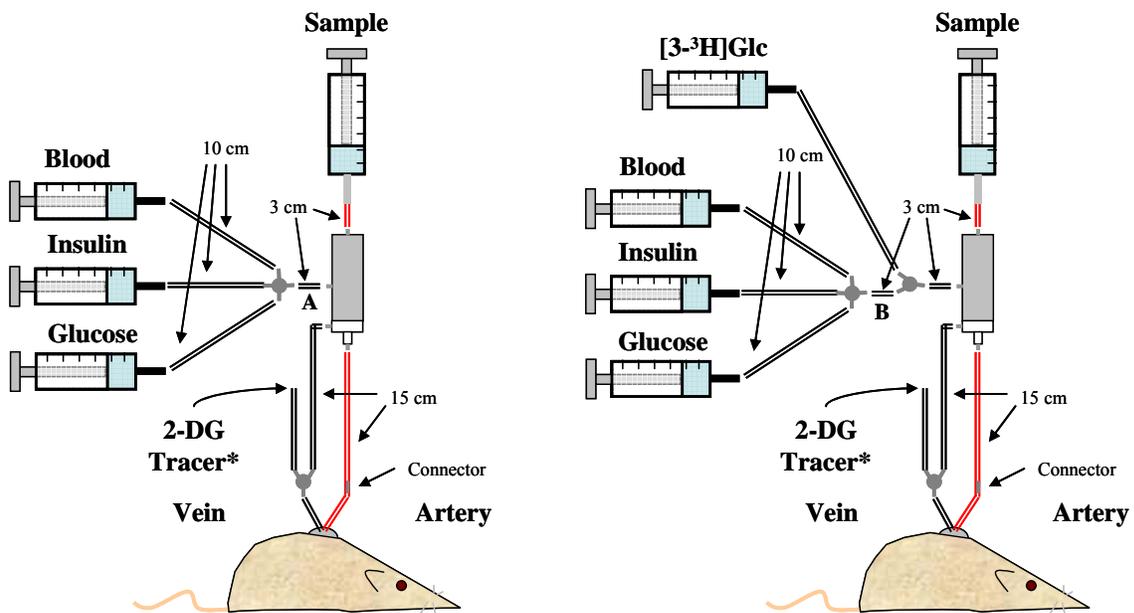
Pumps	Microrenathane tubing
Swivel stand	(0.033 in OD)
Dual channel swivel	Blunt 1.0 ml syringes
3- and 4-way connectors	1.5 ml tubes
Plastic container or restrainer	0.5 ml tubes with dried EDTA (20 μ l 0.1M)
Hematocrit tubes and clay	Ice
Saline	Timer
Heparinized saline (10 U/ml)	Glucose analyzer

1. Getting Started



1. Weigh mouse and start fast (suggested starting time between 7:00 and 8:00 AM) by placing mouse in a plastic container with fresh bedding.
2. Set up microrenathane tubing to swivel and connectors as needed (see Figure 5).
3. Prepare donor blood (see *Donor Blood Preparation*).
4. Prepare tracers, if needed (see *Tracer Preparation*).
5. If using [$3\text{-}^3\text{H}$]glucose, draw tracer infusate into a 1 ml syringe and place it securely into the pump slot.
6. Fill the arterial line with heparinized saline. Leave the sampling syringe connected to the top of the swivel. Fill the venous line with non-heparinized saline (or tracer, if using [$3\text{-}^3\text{H}$]glucose). Plug the free end of the 3 cm microrenathane tubing going into the venous port of the swivel (**A** in Figure 5) or the free end of the 3 cm tubing going into the 3-way connector if using [$3\text{-}^3\text{H}$]glucose (**B** in Figure 5) with a stainless steel plug. Filling these lines prevents infusion of air into the mouse.
7. If using [$3\text{-}^3\text{H}$]glucose, weigh the mouse and hook it up to the microrenathane tubing ~100 minutes prior to clamp start as shown in Figure 5. At this point the mouse can either remain in the plastic container or be placed in a restrainer (see *Cut Tail Sampling* below).

8. Begin primed-continuous tracer infusion at least 90 minutes prior to clamp start. We typically use a 1 μCi prime followed by a 0.05 $\mu\text{Ci}/\text{min}$ continuous infusion.
9. Prepare Protocol sheets using mouse weight, insulin dose, sample times and volumes (see Forms on following pages).
10. Prepare insulin (see *Insulin Preparation*).
11. Once donor blood and insulin are prepared, draw glucose infusate (5%, 20%, or 50%), insulin infusate, and donor blood into separate 1 ml syringes and place them securely into the pump slots.
12. Connect 10 cm microrenathane tubing to each infusate syringe, and connect tubing to a 4-way connector (see Figure 5). Fill each line with glucose, insulin, and donor blood infusates up to the 4-way connector.
13. Once the last basal sample is taken (see *Arterial Sampling*), connect the 4-way connector to the 3 cm microrenathane tubing connected to the infusion port of the swivel (or the 3 cm tubing connected to the 3-way connector if using $[3\text{-}^3\text{H}]\text{glucose}$), and begin infusion of donor blood.
14. Once donor blood reaches the mouse, start study by beginning insulin and glucose infusion (and increasing tracer infusion if using $[3\text{-}^3\text{H}]\text{glucose}$).



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Figure 5. Scheme illustrating the connection of catheters, dual channel swivel, and infusates in relation to the mouse when clamping without (left panel) or with (right panel) $[3\text{-}^3\text{H}]\text{glucose}$. Diagram is not to scale. *This port is generally for 2-deoxyglucose (2DG) tracer bolus administration, but can be used for other infusates as well. **A** and **B** indicate the 3 cm microrenathane tubing filled with non-heparinized saline and capped with a stainless steel plug (see step 6).

Notes on Proper Clamping Technique

1. If a priming dose of insulin is required, calculate the dose needed to reach the target steady state insulin concentration, which is empirically determined. For example, with a $4 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ insulin infusion, steady state insulin concentration will be $\sim 80 \text{ }\mu\text{U}/\text{ml}$. Assuming the insulin volume of distribution is 20% of the body weight, then for a 20 g mouse the priming dose needed to achieve the target insulin concentration is 16 mU/kg. However it has been our experience that a priming dose is not required when using an insulin infusion of $4 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and performing the clamp for 2 h.
2. Increasing the rate of [$3\text{-}^3\text{H}$]glucose tracer infusion at the start of the clamp (see Step 14 above) is done to account for the increase in glucose turnover that occurs during the clamp period. Otherwise, the glucose specific activity during the clamp would fall compared to the basal specific activity. The magnitude of the increase in tracer infusion should be equal to the magnitude of the increase in glucose turnover. Another option is, instead of increasing the tracer infusion, tracer can be added to the glucose infusate so that the specific activity of the infusate approximates that in the pre-clamp blood. The latter technique is superior if it is known ahead of time that the contribution of the liver to total glucose appearance will be low or zero.
3. The goal of the clamp is, as one approaches the steady state period ($t = 80$ to 120 min), to maintain glucose levels within the predetermined range while avoiding large changes in the glucose infusion rate. If this has not been achieved prior to the steady state period (or prior to the 2-deoxyglucose bolus, see below), stop the clock. While the clock is stopped, measure blood glucose every 5 minutes and make changes to the glucose infusion rate as needed. Once the target glucose level has been attained with minimal changes to the glucose infusion rate, restart the clock and continue with the clamp.
4. For 2-deoxyglucose, use a 1 ml blunted insulin syringe to administer the $48 \text{ }\mu\text{l}$ bolus (see *Tracer Preparation*), as this syringe has a small dead space. Give the bolus just prior to the steady state period (we administer the bolus at $t = 120$ min), and chase it with $\sim 20 \text{ }\mu\text{l}$ of saline to clear the line. Momentarily clamp off other infusion lines while the bolus is being given as it may back up into these lines. Once the bolus of 2-deoxyglucose has been given, the clock cannot be stopped.

3. **Blood Sampling**

a. **Arterial Sampling**

1. The sampling syringe attached to the top of the swivel should be filled with ~ 500 ul heparinized saline.
2. Pull back slowly on the plunger to draw blood into the syringe. Draw up ~ 20 – 40 ul of blood into the syringe.
3. Clamp the 3 cm microrenathane tubing connected to the sampling syringe either by pinching with a hand or with clamps with silicon tubing over tips. Clamps can also be used on the 15 cm tubing connected to the bottom of the swivel.
4. Once the arterial line is clamped, remove the sampling syringe and set aside, leaving it vertical such that the ~ 20 – 40 ul of blood does not mix with the heparinized saline.
5. To take glucose samples, release the clamp on the arterial line and allow blood to flow out of the free end of the tubing. A drop of blood will form that can be used on cuvettes or testing strips.
6. To take blood samples for analysis of plasma metabolites:
 - Insert a blunt syringe into the free end of the arterial line prior to releasing the clamp.
 - Release the clamp and draw the desired volume of blood into the syringe.
 - Clamp the arterial line and remove the syringe.
 - Dispense the blood from the syringe into a 0.5 ml EDTA tube and mix by “flicking” the tube.
 - Centrifuge for 1 min at 16,000 g. Transfer the plasma to an appropriately labeled 1.5 ml tube. Plasma can be temporarily stored on ice but should be permanently stored at –20°C.
 - While the blood is being centrifuged, insert the sampling syringe back into the arterial line. Pull back slightly on the plunger to draw any air bubbles into the sampling syringe. Very slowly, give back the ~ 20 – 40 ul of blood originally taken such that the arterial line becomes lightly red to clear.

b. **Cut Tail Sampling**

Bleeding from the cut tail is an alternative to arterial sampling if carotid artery catheterization proves too challenging due either to the surgeon’s skill level or an intolerance of a given mouse to surgery. The advantage of using the cut tail is that surgery is less involved (for both surgeon and mouse). The disadvantage is that obtaining more than small blood volumes is stressful for the mouse and it is difficult to obtain blood samples at pre-determined intervals. The latter depends on how effectively blood drips from the wound.

Supplies:

Plastic restrainer
Scalpel
Tape (very adhesive, such as duct tape)
Platform (~ 1 cm high)
Silk suture (optional)

Procedures for obtaining blood from the cut tail:

1. Place mouse in restrainer prior to the equilibration period and lock the plastic doughnut into place about halfway down the restrainer tube.
2. Place the restrainer with mouse on the ~ 1 cm high platform near the edge of the platform.
3. Tape down the tail near the edge of the platform so that 2-3 cm of the tail hangs off the edge of the platform.
4. Hook the infusion lines as necessary.
5. When ready to start sampling, brace the 2-3 cm of tail hanging off the edge with a hard surface and cut off ~ 1 cm of the tail tip with a scalpel. (Note consult with your IACUC it may require cutting the tail doing this under gas anesthesia)
6. The amount of bleeding will depend on individual mice. In order to obtain larger samples, manual “milking” of the tail is required.
7. “Milking” of the tail is more efficient from the dorsal side of the tail. If the mouse twists such that the ventral side of the tail is up, the tail must be manually twisted back so that the dorsal side is up.
8. Begin by pressing the base of the tail with a finger and pushing forward towards the tape. Repeat this several times quickly.
9. Then push one last time all the way through the tape and towards the cut end of the tail. A large drop of blood should appear at the end of the tail. Repeat these steps as necessary.
10. If the mouse is a profuse bleeder, ligate the end of the tail with suture.

3. Preparation of Infusates

Donor Blood Preparation:

1. Collect ~ 1 ml of blood from donor mouse in 0.5 ml EDTA tubes.
2. Centrifuge blood (1 min at 16,000 g) and save plasma for preparation of insulin (see below).
3. Resuspend red blood cells (RBC) with heparinized saline.
4. Centrifuge (1 min at 16,000 g), discard supernatant, and resuspend RBC with an equal volume of heparinized saline. Transfer resuspended RBC (donor blood) to a 1.5 ml tube

Insulin Preparation:

1. Using the plasma obtained from donor mice, make a 3% plasma solution in saline by placing 6 ml of saline in a test tube, removing 180 μ l and adding 180 μ l plasma.
2. Remove 180 μ l of this 3% saline-plasma solution, place in a tube and add 20 μ l U-100 insulin (making a tenfold dilution to create U-10 solution).
3. Transfer 5 ml of 3% saline-plasma solution to a new test tube.
4. Calculate and add U-10 insulin to achieve desired insulin infusate concentration based on mouse weight, pump infusion rate, and desired insulin infusion rate.

[3-³H]Glucose Infusate Preparation:

1. Dry down 50 μ Ci HPLC-purified [3-³H]glucose per mouse.
2. Reconstitute in 1 ml of saline. Save 20 μ l for standards.

2-Deoxyglucose (¹⁴C or ³H) Bolus Preparation:

1. Dry down 13 μ Ci of 2-deoxyglucose per mouse.
2. Reconstitute in 52 μ l of saline. Save 4 μ l for standard. The remaining 48 μ l is given as the 2-deoxyglucose bolus.

4. Analysis of Radioactivity (when applicable)

Plasma Glucose and 2-Deoxyglucose Radioactivities

Reagents and supplies:

Saline
Saturated benzoic acid (3.4 g/l)
0.3 N Ba(OH)₂ diluted 1:3 with ddH₂O. Prepared fresh.
0.3 N ZnSO₄ diluted 1:3 ml with ddH₂O. Prepared fresh.
1.5 ml tubes
20 ml borosilicate scintillation vials

Processing ¹⁴C- or ³H-2-deoxyglucose standards:

1. Add 196 µl ddH₂O to 4 µl of standard (saved from bolus of 2-deoxyglucose) and vortex.
2. Pipette 50 µl diluted standard into a 1.5 ml tube and add 950 µl ddH₂O.
3. Pipette 100 µl into scintillation vial.
4. Add 900 µl ddH₂O and 10 ml scintillation fluid.
5. Measure radioactivity in a liquid scintillation counter.

Processing [3-³H]glucose standards:

1. Dilute tracer standard 1:200 in saturated benzoic acid (5 µl in 995 µl).
2. Add 10 µl of this diluted standard directly to each of three scintillation vials labeled CS. Add 990 µl ddH₂O and cap.
3. Add 10 µl of the diluted standard directly to each of three separate scintillation vials labeled CSE and set aside for drying later (see *Plasma processing* below).
4. Add 10 µl of the diluted standard to each of three 1.5 ml tubes. To each tube add 10 µl saline + 100 µl diluted Ba(OH)₂ + 100 µl diluted ZnSO₄.
5. Vortex and spin down for 5 minutes at 16,000 g.
6. Transfer 100 µl of the supernatant to each of three scintillation vials labeled CRS and set aside to dry later (see *Plasma processing* below).

Plasma processing:

1. Add 10 μl of plasma sample and 10 μl saline to a 1.5 ml tube.
2. Add 100 μl diluted $\text{Ba}(\text{OH})_2$ solution and 100 μl diluted ZnSO_4 (add $\text{Ba}(\text{OH})_2$ prior to ZnSO_4).
4. Vortex and centrifuge (1 min at 16,000 g).
5. Pipette 100 μl of supernatant into scintillation vial.
6. Dry in oven with a trap for $^3\text{H}_2\text{O}$. Include standards (CSE and CRS) for drying at this time.^{a,b}
7. Add 1 ml ddH₂O and 10 ml scintillation fluid.
8. Measure radioactivity in a liquid scintillation counter.
 - a. To determine $^3\text{H}_2\text{O}$ accumulation, a measure of glycolytic rate, pipette 50 μl of the supernatant into a second set of vials and do not dry. $^3\text{H}_2\text{O}$ will be the difference in radioactivity between these non-dried samples and the dried samples (correcting for the difference in the volume of supernatant used).
 - b. Analysis of samples containing only radioactive 2-deoxyglucose does not require that they be dried. Simply add 900 μl ddH₂O and 10 ml scintillation fluid to the 100 μl of supernatant in the scintillation vial.

Tissue 2-Deoxyglucose Phosphate Radioactivity

Reagents and supplies:

0.3N $\text{Ba}(\text{OH})_2$
0.3N ZnSO_4
0.5% perchloric acid
Liquid nitrogen
Ice
20 ml borosilicate scintillation vials
5 ml and 13 ml large polypropylene tubes w/ caps
1.5 ml tubes
5N KOH and 10 N HCl
Forceps
Homogenizer
pH meter

1. Keep tissues and 5 ml tubes in liquid nitrogen and make sure that the end of the forceps that will come in contact with tissue is cooled in liquid nitrogen.

2. Weigh each tissue sample (~60 mg), transfer to 5 ml tube in liquid nitrogen and record weight. *Note: you will not obtain 60 mg in all tissues (e.g. soleus muscle). In those cases, simply weigh out the entire amount of tissue obtained.*
3. For each sample, place on ice the 5 ml tube containing the tissue and homogenize in 1.5 ml 0.5% perchloric acid. Keep the homogenized sample on ice while the remaining tissues are homogenized.
4. Centrifuge tubes for 20 min at 2000 g at 4°C.
5. Place tubes on ice and transfer 1.25 ml of the supernatant to 13 ml tubes. If you are unable to extract 1.25 ml of supernatant, note the volume of supernatant you are able to extract.
6. Neutralize each sample (pH ~7.5) using KOH and HCl. Record the total volume of KOH and HCl used to neutralize each sample.
7. Centrifuge tubes for 10 min at 2000 g at 4°C.
8. Label two scintillation vials (A and B) for each tissue, and number consecutively

For the A vials (measurement of 2-deoxyglucose phosphate and 2-deoxyglucose)
Add 125 µl of neutralized supernatant and 875 µl ddH₂O

For the B vials (measurement of 2-deoxyglucose only)
Pipette 250 µl of neutralized supernatant into 1.5 ml tubes
Add 125 µl of stock 0.3N BaOH
Add 125 µl of stock 0.3N ZnSO₄
Centrifuge at 16,000 g for 5 minutes
Add 250 µl of supernatant and 750 µl ddH₂O to vials
9. Add 10 ml of scintillation fluid to each vial, shake and measure radioactivity in liquid scintillation counter.
10. 2-deoxyglucose phosphate is the difference between radioactivity in A and B.

5

Considerations in Designing Insulin Clamp Experiments in the Conscious Mouse

There are numerous factors to consider when designing an insulin clamp to study the conscious mouse. Most of these factors apply to clamping any organism while others are specific to the mouse. Below is a list of general considerations. The pages that follow summarize experiments conducted in the Vanderbilt MMPC investigating the impact of select experimental parameters.

Considerations

- Strain
- Genotype
- Observable phenotype
- Gender
- Breeding and other husbandry issues
- Diet
- Weight and body composition
- Age
- Insulin dose
- Tracer application
- Sampling site
- Blood sampling volume
- Volume infused
- Main tissues and metabolic pathways of interests

Please see the following publications for experiments by the Vanderbilt MMPC addressing some of the issues listed above:

Ayala JE, Bracy DP, McGuinness OP, Wasserman DH. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes*. 55(2): 390-7, 2006.

Berglund ED, Li CY, Poffenberger G, Ayala JE, Fueger PT, Willis SE, Jewell MM, Powers AC, Wasserman DH. Glucose metabolism in vivo in four commonly used inbred mouse strains. *Diabetes*. 57(7):1790-9, 2008.

McGuinness OP, Ayala JE, Laughlin MR, Wasserman DH. NIH Experiment in Centralized Mouse Phenotyping: The Vanderbilt Experience and Recommendations for Evaluating Glucose Homeostasis in the Mouse. *Am J Physiol Endocrinol Metab* 297(4):E849-855, 2009.

Wasserman DH, Ayala JE, McGuinness OP. Lost in translation. *Diabetes* 58(9):1947-50, 2009.

Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, McGuinness OP; for the NIH Mouse Metabolic Phenotyping Center Consortium. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech* 3 (9-10):525-534, 2010.

6

Use of Tracers to Assess Insulin Action

Isotopic tracers can be used in conjunction with the insulin clamp technique to assess sites of insulin action and insulin's effects on specific metabolic pathways. In this section, special reference is made to two isotopes commonly used to assess glucose metabolism during an insulin clamp. These are [3-³H]glucose which is used to measure whole body glucose turnover and 2-[³H]deoxyglucose which is used to measure tissue-specific glucose uptake. The principles introduced in this section, however, are of general applicability to other isotopes.

Use of Tracers to Assess Insulin Action

Living systems are in a constant state of change. Arterial glucose concentration over the course of a day changes very little. This constancy is deceiving in a sense. Although blood glucose is maintained within narrow limits, glucose molecules that comprise the blood glucose pool are continually being exchanged with body tissues. Arterial glucose is in a dynamic steady state. The rate of tissue-blood glucose exchange varies with physiological conditions such as exercise and insulin stimulation. The rate of glucose exchange is also affected by pathological conditions, most notably diabetes.

If isotopic glucose (radioactive or stable) is injected or infused into the body, it disappears in a finite time period. In a steady state, the rate that isotopic glucose disappears is directly related to the rate at which glucose is removed. The use of isotopic glucose to measure glucose turnover requires that the isotope is:

1. Nontoxic and doesn't affect glucose metabolism.
2. Spread evenly throughout the body (well mixed).
3. Not recycled back to glucose once it is removed (no recirculation, irreversible loss).
4. Handled equivalently to that of nonlabeled glucose. (no “isotope effect”).

If the ratio of isotopic glucose to non-isotopic glucose is measured and the assumptions hold then the mass glucose turnover rate can be quantified. The goal of this section of the course is to explain how tracers can be used to assess the kinetics of substrates *in vivo*.

Glossary of Terms

Turnover

Turnover is the renewal rate of a substance "A" in a given system. This renewal can take place in two possible ways:

1. The substance "A" can be synthesized and metabolized in the same tissue.
2. The substance "A" can be synthesized at a site distant from its site of metabolism.

Note: that the term turnover has an unequivocal meaning only when "A" is in a steady state. Units: mg/min, μ moles/min, mg/kg/min

Dynamic steady state (DSS)

DSS exists when the mass of a substance in a defined system remains constant for a period of time. When we are in a steady state then the rate of production or appearance (R_a) of a substance equals, by definition, the rate of removal (R_d) of the substance.

$$(R_a=R_d)$$

Note: we are also assuming that the volume of the pool and the dynamics of mixing in the pool in which that substance is dissolved remain constant.

Pool

Pool is the space in which the substance of interest is distributed. This space is assumed to be well-mixed.

Tracee

Tracee is the substance whose turnover is measured.

Tracer

Is isotopically labeled (radioactive or stable isotope) form of the tracee.

The position of the radiolabel in the molecule is important in interpretation of results.

Specific activity (SA)

SA is the ratio of the amount of tracer to tracee in the pool.

The units are usually expressed as dpm/mmol, dpm/g or cpm/g

Where dpm=disintegrations per min; cpm=counts per min

If stable isotopes are used "enrichment" is defined as the ratio of tracer to tracee.

Fractional turnover rate (k)

k is the fraction of the tracee that is replaced per unit time assuming a single pool.

Half life ($t_{1/2}$)

Half life is the time it takes to replace one half of the original components of the pool.

k and $t_{1/2}$ are related by the following equation:

$$F = e^{-kt}$$

Where F= fraction of the original tracee that remains at any time (t). When $F=1/2$ then $t=t_{1/2}$. If we solve for $t_{1/2}$ in terms of k.

$$1/2 = e^{-kt_{1/2}}$$

$$\ln(1/2) = -kt_{1/2} \quad -\ln(2) = -kt_{1/2}$$

$$\frac{\ln(2)}{k} = t_{1/2}$$

Example: if $k=0.1/\text{min}$ then $t_{1/2}=6.9 \text{ min}$

Volume of distribution (V_d)

V_d is an empirically derived pool size. V_d is usually very close to the true pool size only if the model, which best describes the kinetics of the pool is one compartment. Since the body usually cannot be considered a single compartment, the V_d only approximates the pool size. The units are usually ml/kg.

Clearance

Clearance is the ratio of R_d and the tracee concentration (units: ml/kg/min or ml/min). It is an index of the avidity with which the peripheral tissues want to take up the tracee. Note that the ratio of clearance and V_d is equal to k if you assume a single compartment.

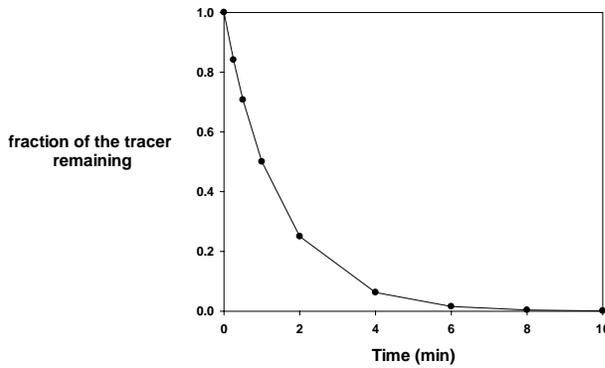
$$k = \frac{\text{Clearance}}{V_d}$$

Methodological Approaches to Measuring Turnover

1. Bolus Method

R_t is the rate at which glucose enters and leaves V_d . The fraction of this glucose pool that is “turned over” per unit time is called the fractional turnover rate (k). It has units $=\text{time}^{-1}$. If $k=1.0 \text{ min}^{-1}$ then 100% of this mass of glucose M is replaced per minute. If $k=0.5 \text{ min}^{-1}$ then 50% of the pool is replaced per min.

To assess the rate of removal of glucose from the pool one takes advantage of the fact that the removal mechanism cannot distinguish between tracer and tracee. By adding an isotopic glucose tracer at time=0 and following its removal rate, the rate of removal of glucose can be assessed. Suppose an amount of isotopic glucose defined as M^* is injected. If M^* mixes instantly in the pool, the cells cannot distinguish it from the tracee and the tracer is irreversibly disposed of, then it can be a good index of the glucose utilization rate. The concentration of the tracer will decrease in an exponential fashion.



In the adjacent figure it is assumed that the half-life of glucose is one minute and we take samples from the plasma pool at designated times.

<u>Time (min)</u>	<u>% remaining</u>
1	50
2	25
3	12.5

Note: the curve is exponential and follows the equation $F=e^{-kt}$. Since $t_{1/2}=1$ min then $k=\ln 2=0.693 \text{ min}^{-1}$.

To determine $[M^*_t]$, the amount of tracer remaining at any time multiply both sides of the equation by M^* (the quantity of tracer added).

$$M^* \cdot F = M^* e^{-kt} = M^*_t$$

note $M^*_t = M^*$ when $t=0$

Since we are in a DSS, V and M remain constant, we can divide both sides of the equation by the constant M .

$$\frac{M^*_t}{M} = \frac{M^* e^{-kt}}{M}$$

M^*_t/M is equal to the SA:

$$SA_t = SA_0 e^{-kt}$$

where SA_0 is the specific activity at time = 0 which is obtained by extrapolating back to zero time. Since $SA_0 = M^*/M$ and M^* is known (the amount of tracer we injected) we can calculate M . k is calculated by determining the slope of the line plotting $\ln(SA_t)$ vs. time.

R_t is equal to the product of k and M . $R_t = k \cdot M$

Assumptions:

1. DSS
2. Tracer acts like tracee (in addition it must not be contaminated tracer)
3. Mass of tracer is negligible compared to the mass of the tracee in the pool
4. Instantaneous mixing of tracer and tracee
5. One compartmental model (homogeneity of the pool).
6. No recirculation of tracer
7. Loss of tracer reflects irreversible loss of tracee

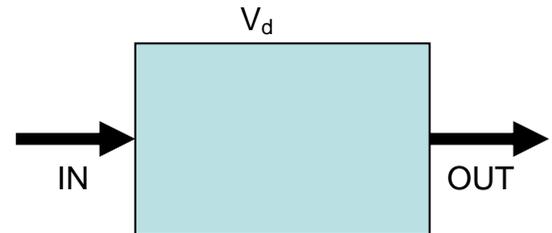
What is done in practice? In practice a series of plasma samples (dpm/ml plasma) are collected over time after the bolus injection. If the data are plotted as the natural log of dpm over time then the slope is equal to k . The y-intercept is dependent upon the V_d and the total amount of radioactivity injected (M^*). [V_d (ml) = (dpm injected) / (dpm/ml at time zero)]. Once the y-intercept is determined V_d can be calculated. k and V_d are then used to calculate substrate turnover and clearance. While the bolus technique seems easy it has its limitations. If the bolus technique is used to estimate R_t a number of samples over time must be taken to obtain an accurate curve fit. Only one estimate of R_t is obtained.

2. Constant Infusion Method

Steady state conditions

$$\text{In} = \text{Out}$$

Tracer entry is equal to the rate of tracer exit in a steady state. The ratio of the rates of tracer and tracee exit the system is proportional to the ratio of the tracer to the tracee (i.e. specific activity) since the body treats them equivalently.



$$R_d^* = \frac{A^*}{A} \cdot R_d$$

$$R_a^* = I = R_d^*$$

$$R_a = R_d = R_t$$

$$SA_a = \frac{A^*}{A}$$

Where

I = tracer infusion rate

A^* = tracer concentration

A = tracee concentration

R_a = rate of tracee appearance

R_d = rate of tracee disappearance

R_a^* = rate of tracer appearance = tracer infusion rate = I

R_d^* = rate of tracer disappearance

SA_a = specific activity of tracee

$$\text{Therefore } \frac{SA}{R_t} = I = \frac{A^*}{A} R_t$$

$$\text{simplify } R_t = \frac{I}{SA}$$

Note that in a steady state R_t is independent of the size or complexity of the pool.

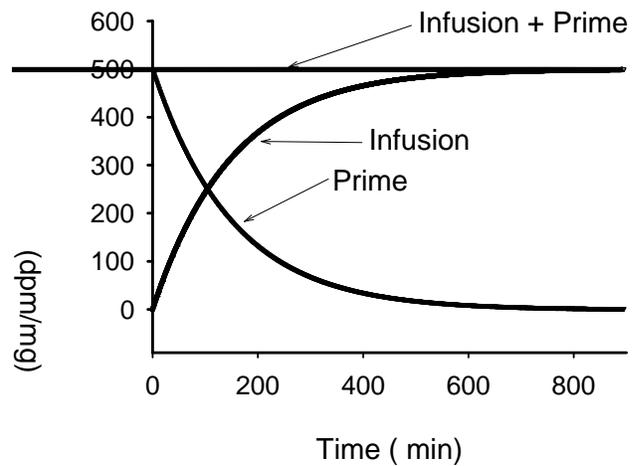
Assumptions:

1. Steady state
2. Tracer acts like tracee
3. No recycling
4. Loss of tracer reflects irreversible loss of tracee
5. Tracer is infused in tracer amounts relative to the amount of tracee
6. The SA of the pool one from blood samples are obtained is equal to the specific activity at the site of utilization.

The strength of this approach is that as long as one is in a DSS one can get multiple estimates of R_t by taking serial samples of specific activity in the plasma pool.

3. Priming the Pool

The goal is to prime the tracer pool to reach a steady state in as short a time as possible during a constant tracer infusion. If a bolus of tracer is given, the peak SA occurs right after the tracer is injected (SA_0). The SA will fall in an exponential manner following the equation $SA_t = SA_0 e^{-kt}$. If a constant tracer infusion is given the SA gradually rises until a steady state is reached. In the adjacent figure SA at $t = \infty$ is 500 dpm/mg. The rate of rise in SA will follow the equation $SA_t = \left(\frac{I}{M \cdot k}\right)(1 - e^{-kt})$



[in the adjacent figure this is the “infusion” line]. Note that when $t = \infty$ the equation simplifies to the steady state equation.

$SA = I / (M \cdot k)$. This is a rearrangement and substitution of the equation $R_t = I / SA$ where $M \cdot k$ was substituted for R_t ; $M \cdot k = R_t$.

The goal when priming the pool is to have $SA_0 = SA_{\infty}$. Thus at any time t , $SA_t = SA_0$. For this to be true the sum of the nonsteady state equations for the prime and constant infusion must equal the steady state equation:

$$SA_0 e^{-kt} + \left(\frac{I}{M \cdot k}\right)(1 - e^{-kt}) = \frac{I}{M \cdot k}$$

The equation can be simplified to:

$$SA_0 \left(\frac{M}{I}\right) = \frac{1}{k}$$

A sound estimate of the prime (P) required to fill the system can be obtained from the preceding relationship. Since $SA_0 \cdot M$ is equal to the amount of prime this equation simplifies to:

$$\frac{P}{I} = \frac{1}{k}$$

k is the fraction of the pool that turns over per unit time which can be estimated if one can approximate R_t and V_d . Thus, the appropriate prime (P) can be calculated once an appropriate constant tracer infusion rate (I) is chosen and k is estimated.

Examples of values in mouse and human:

	units	human	mouse
V_d	ml/kg	200	200
R_t	mg·kg ⁻¹ ·min ⁻¹	2	15
[glucose]	mg/dl	100	150
clearance	ml·kg ⁻¹ ·min ⁻¹	2	10
k	1/min	0.01	0.05
$t_{1/2}$	min	69.31	13.86
k^{-1}	min	100	20
I	dpm·kg ⁻¹ ·min ⁻¹	1000	1000
SA	dpm/mg	500	66.67

Based on the values above for human and mouse tracer is infused at a constant rate (e.g. 1,000 dpm·kg⁻¹·min⁻¹) the amount of prime required is equal to the amount of tracer you would infuse in 100 min in the human (e.g. 100,000 dpm/kg) and in the mouse it would be ~ 20 min (e.g. 20,000 dpm/kg). P/I is not constant for each species; k can vary. If the human is diabetic and the glucose concentration is 200 mg/dl with comparable R_t (2 mg·kg⁻¹·min⁻¹) you will have to give a larger bolus (200 min; 200,000 dpm/kg). Since the exact R_t in any given study will not be known beforehand, historical data and experience can be used to gain a reasonable approximation.

Note: V_d is unique for each tracee. The V_d for glucose, fatty acids, or amino acid are not the same.

4. Choosing the Appropriate Tracer

The most commonly used radioactive tracer for glucose is [3-³H]glucose. When [3-³H]glucose is metabolized it exchanges ³H with H₂O when it passes through the triose phosphate step in glycolysis. Another tracer that is used is [6-³H]glucose. The advantage of these tracers is that they are not recycled into glucose (via gluconeogenesis). Note [6-³H]glucose is metabolized to [³H]lactate which can accumulate in plasma and will have to be removed during analysis along with ³H₂O. [³H]lactate can be taken up by the liver, however ³H is lost to H₂O before it is converted to glucose. In contrast, [¹⁴C]glucose does recycle and its use will lead to an underestimation of glucose turnover. [2-³H]glucose has been used but this is not a good tracer since in the liver futile cycling between glucose and glucose 6-P pool will lead to a rapid loss of ³H. This will result in an overestimate of glucose flux to that futile cycling is occurring. In fact some investigators infuse both [2-³H]glucose and [3-³H]glucose and measure the difference in the two estimates of R_t as a measure of hepatic futile cycling.

5. Non-Steady State Analysis of Tracee Flux

If a non-steady state exists then either the amount of tracer (M^*) or tracee (M) in the pool is changing. This can be expressed mathematically by taking the derivative of both sides of the equation $M^* = SA \cdot M$.

$$1) \quad \frac{\partial M^*}{\partial t} = SA \frac{\partial M}{\partial t} + M \frac{\partial SA}{\partial t}$$

$$2) \quad R_a - R_d = \frac{\partial M}{\partial t} = V_d \cdot \frac{\partial A}{\partial t}$$

Note the implication of equation 2 is that when R_a is not equal to R_d ($IN \neq OUT$) then the mass of the tracee (e.g. glucose) will change (ie. $dM/dt \neq 0$).

$$3) \quad \frac{\partial M^*}{\partial t} = I - R_d \cdot SA$$

The implication of this equation is that when the tracer infusion rate (I) is not equal to the rate the tracer is leaving the body ($IN \neq OUT$) then the mass of the tracer (e.g. [3H]glucose) will change ($\partial M^*/\partial t \neq 0$).

Place equations 2 and 3 in equation 1:

$$I - R_d \cdot SA = SA(R_a - R_d) + M \frac{\partial SA}{\partial t}$$

Solve for I:

$$I = R_d \cdot SA + SA(R_a - R_d) + M \frac{\partial SA}{\partial t}$$

Simplify:

$$I = R_a \cdot SA + M \frac{\partial SA}{\partial t}$$

Solve for R_a :

$$R_a = \frac{I - M \frac{\partial SA}{\partial t}}{SA} \quad \text{since } M = V_d \cdot A \text{ then}$$

$$R_a = \frac{I - V_d \cdot A \frac{\partial SA}{\partial t}}{SA}$$

since $R_a - R_d = V_d \cdot \frac{\partial A}{\partial t}$ the following relationship can be calculated:

$$R_d = R_a - V_d \cdot \frac{\partial A}{\partial t}$$

Note if in a steady state $\partial SA/\partial t = 0$ then $R_a = I/SA$.

$$M = V_d \cdot A$$

where V_d =volume of distribution of A
A= concentration of tracee

The two equations (see below) are used to calculate R_a and R_d over a time interval. This contrasts with steady state equations where R_t is calculated at a specific point in time.

$$R_a = \frac{I - V_d \cdot \bar{A} \frac{\partial SA}{\partial t}}{\bar{SA}}$$

Note: in this equation the mean SA and mean A for a given time interval is used (depicted as \bar{SA} and \bar{A})

$$R_d = R_a - V_d \cdot \frac{\partial A}{\partial t}$$

These two equations assume a single compartment of known size (V_d).

Example:

time	[glucose]	[³ H glucose]	I	SA	R _t	V _d	time interval	dA/dt	dSA/dt	R _a	R _d
min	mg/dl	dpm/ml	dpm·kg ⁻¹ ·min ⁻¹	dpm/mg	mg·kg ⁻¹ ·min ⁻¹	ml/kg	min	mg·ml ⁻¹ ·min ⁻¹	dpm·mg ⁻¹ ·min ⁻¹	mg·kg ⁻¹ ·min ⁻¹	mg·kg ⁻¹ ·min ⁻¹
-10	100	1000	10000	1000	10	200	(-10 - 0)	0.00	0.00	10.00	10.00
0	100	1000	10000	1000	10	200	(0 - 20)	0.01	-0.17	12.04	10.04
20	120	800	10000	667	15	200	(20 - 40)	0.00	0.00	15.00	15.00
40	120	800	10000	667	15	200	(40 - 60)	-0.01	0.00	15.00	17.00
60	100	667	10000	667	15	200					

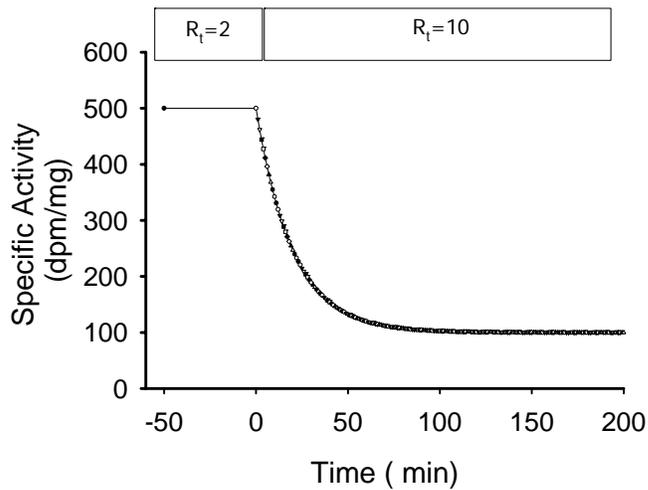
A steady state exists between -10 and 0. Thus, R_t at -10 and 0 min is equal to the R_a and R_d for that interval. For interval 0 to 20 min, glucose levels rose but [³H]glucose concentration and SA fell. One can see that steady state equations give an overestimate. By calculating R_a and R_d using non-steady state equations it can be seen that glucose levels increase because R_a rose, while R_d did not increase very much. After an initial non-steady state interval glucose concentration and SA stabilize such that $R_a=R_d=R_t$. Between 40 and 60 min the steady state equation predicts that R_t did not change even though glucose falls (of course this cannot be true). However with non-steady state equations, it is apparent that the fall in glucose is due to a large increase in R_d , while R_a does not increase. It is evident that both the SA and the tracee concentration must be constant for the estimate of R_t to be valid.

Assumptions:

1. Well mixed *single* pool
2. Irreversible disposal
3. Mass of tracer is negligible to the mass of the tracee
4. Tracer behaves like tracee
5. The SA of the pool that is sampled is equal to the SA at the site of utilization.

6. Implications of Non-Steady State

During an insulin clamp, total glucose flux increases and SA decreases (remember $\uparrow R_t = \frac{I}{\downarrow SA}$). Endogenous glucose production is calculated as the difference between



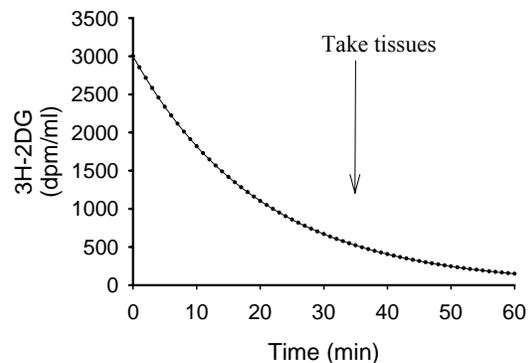
R_a and the exogenous glucose infusion rate. It takes more than 80 min to reach a new steady state in the human. If steady state calculations are used prior to 80 min to estimate total glucose turnover the total R_a would be underestimated. The underestimation of R_a would lead to an overestimate of the suppression of endogenous glucose production by insulin. In fact, negative estimates of endogenous

glucose production, which are theoretically impossible, may be calculated. If non-steady state equations are used, more accurate estimates of R_a as well as R_d , are obtained. However, non-steady state equations can yield inaccurate results if the assumption of a single well-mixed pool is not entirely valid and changes in the rate of glucose infusion are required. The best way to perform the clamp is to: 1) minimize changes in SA and 2) take multiple SA measurements so that non-steady state equations can be optimally applied.

Changes in SA can be minimized by two approaches. One can infuse extra tracer when the clamp is started to minimize the fall in SA. Alternatively, tracer can be mixed with exogenous glucose to obtain a SA identical to basal SA and vary the constant tracer infusion rate as you expect endogenous glucose production to change. If a decrease in endogenous glucose production of 50% is expected, decrease the constant tracer infusion rate by 50%. The difficulty with the latter approach is that the system being studied must be well-defined in advance.

7. Estimating Tissue Specific Glucose Uptake

Labeled 2-deoxyglucose has been validated as a tracer and been used to quantitate rates of glucose uptake in a variety of tissues (e.g. skeletal muscle, heart, brain). During the experiment a bolus of labeled 2-deoxyglucose (2-DG) is given and a series of blood samples are taken over the course of 20 to 40 min. Tissues are then collected to determine how much 2-deoxyglucose-6-phosphate (2-DGP) has accumulated in the tissues of interest. Tissue glucose clearance is quantitated by dividing tissue 2-DGP radioactivity by the integrated area of the plasma radioactivity - time curve of 2-DG. This is then normalized to a rate of glucose uptake by multiplying by the average glucose concentration during the decay period.



The tissue-specific clearance of [2-³H]DG, or K_g , and the metabolic index, or R_g are calculated as described below.

$$K_g = \frac{[2-^3H]DGP_{muscle}}{AUC[2-^3H]DG_{plasma}}$$

$$R_g = K_g \cdot Glucose_{plasma}$$

where [2-³H]DGP is the [2-³H]DGP_{muscle} radioactivity in the muscle in dpm/g, AUC[2-³H]DG_{plasma} is the area under the plasma [2-³H]DG disappearance curve calculated using the trapezoid method (dpm/ml·min), and Glucose_{plasma} is the average plasma glucose (mM or mg/dl) during the decay period. Typical units for K_g and R_g are ml·100g⁻¹·min⁻¹ and μmol·100g⁻¹·min⁻¹ or μg·100g⁻¹·min⁻¹, respectively.

The assumption is that 2-deoxyglucose can be transported and phosphorylated, but not further metabolized. Thus any 2-DGP that is made by the tissue is trapped. This method is invalid for tissues that contain glucose-6 phosphatase (liver, renal cortex, and possibly intestine) since 2-DGP is not effectively trapped. 2-DG is not an ideal tracer if glucose transporters or hexokinases differentiate between 2-DG and glucose. If this is the case, the clearance rate of 2-DG (K_g) will not equal glucose clearance rate. A factor has been used in some studies to correct for differences in the transport and phosphorylation of 2-DG and glucose. This factor is called the lumped constant (LC). LC in muscle and brain are very close to 1 and do not seem to be affected by insulin.

Suggested Reading:

Kraegen EW, James DE, Jenkins AB, Chisholm DJ: Dose-response curves for in vivo insulin sensitivity in individual tissues in rats. *Am J Physiol* 248 :E353 –E362,1985.

De Boisblanc BP, Dobrescu C, Skrepnik N, Nelson S, Spitzer JJ, Bagby GJ: Compartmentalization of glucose utilization after intravenous vs. intratracheal challenge with LPS. *Am J Physiol* 270:L452-8,1996.

Wolfe RR, Chinkes DL. *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*, 2nd Edition. John Wiley and Sons, Inc., New Jersey. 2004.

8. Example for Calculating Glucose Flux in Mice

The following information is needed to calculate glucose turnover:

- A. **Body weight of mouse (grams)**
- B. **Glucose infusion rate must be recorded throughout the study (mg/kg/min)**
- C. **Tracer infusion rate:**
 1. **A precise pump rate ($\mu\text{l}/\text{min}$)**
 2. **Concentration of tracer in infusate containing $[3\text{-}^3\text{H}]\text{Glucose}$ (dpm/ μl).**

Save some of the infusate and dilute 1:200. Add 10 μl to scintillation vial (in triplicate). Dry down and reconstitute in water and scintillation fluid. This vial is labeled CSE (Chemical Standard Evaporated), you can also pipette some and not evaporate it to see if there is some $^3\text{H}_2\text{O}$ in the tracer (Chemical Standard). A portion of the diluted infusate (10 μl) is processed in an identical manner as the plasma samples (see below). This is the Chemical Recovery Standard (CRS).
- D. **Specific activity of plasma glucose at each time point**
 1. **Plasma glucose concentration (mg/dl plasma):** Note that the blood and plasma concentration may not be the same and that plasma values are needed to calculate glucose fluxes. The Beckman glucose analyzer, which uses plasma, is not practical to use, as it requires taking a large blood sample (~20 μl). The Hemocue (which uses blood) gives a reading very similar to the Beckman which measures “plasma” glucose but still requires ~7 μl blood. Note other blood glucose meters are designed for human not rodent blood and are not accurate enough to assess “plasma” glucose. Vanderbilt MMPC measures the glucose concentration in the $\text{Ba}(\text{OH})_2$ and ZnSO_4 supernatant using an enzymatic assay.
 2. **Plasma $[3\text{-}^3\text{H}]\text{glucose}$ concentration: (dpm/ μl plasma)** Plasma samples will have to be treated with $\text{Ba}(\text{OH})_2$ and ZnSO_4 , evaporated and counted. Multiply plasma radioactivity by the CSE to CRS ratio to correct for dilutions and assay recovery.
- E. **Example calculations**
 1. **Whole Body Glucose Fluxes**

Body weight: 38.3 g
CSE: 5398 dpm (10 μl of diluted 200-fold infusate)
CRS: 2127 dpm (10 μl of infusate added to $\text{Ba}(\text{OH})_2$ and ZnSO_4)
CSE/CRS = 2.54
Pump rate of tracer: 1 $\mu\text{l}/\text{min}$ -120 to 0 min
2 $\mu\text{l}/\text{min}$ 0 to 120 min

Time min	Glucose mg/dl	Plasma [³ -H] glucose dpm	Glucose infusion rate mg/kg/min
0	136	1050	0
80	171	1394	22
90	176	1408	22
100	163	1390	22
110	165	1325	22
120	169	1295	22

$$\text{Tracer infusion rate (dpm/kg/min)} = \frac{\left[\frac{\text{Pump rate} \cdot \text{CSE} \cdot 200}{10} \right]}{\left[\frac{\text{Body weight}}{1000} \right]}$$

$$\text{Specific activity (dpm/mg)} = \frac{\left[\frac{\text{plasma (dpm)}}{10} \cdot \frac{\text{CSE}}{\text{CRS}} \cdot 1000 \right]}{\left[\frac{\text{plasma glucose}}{100} \right]}$$

$$\text{Glucose turnover (R}_t\text{; mg/kg/min)} = \frac{\text{Tracer infusion rate (dpm/kg/min)}}{\text{Plasma glucose specific activity (dpm/mg)}}$$

Endogenous glucose production (Endo R_t) = glucose turnover rate - exogenous glucose infusion rate

Time min	Plasma Glucose mg/dl	Plasma [³ H] glucose dpm	Glucose infusion rate mg/kg/min	Tracer infusion rate dpm/kg/min	SA dpm/mg	R _t mg/kg/min	EndoR _t mg/kg/min
0	136	1050	0	3100679	196103	15.8	15.8
80	171	1394	22	6201358	207062	29.9	7.9
90	176	1408	22	6201358	203200	30.5	8.5
100	163	1390	22	6201358	216601	28.6	6.6
110	165	1325	22	6201358	203970	30.4	8.4
120	169	1295	22	6201358	194633	31.9	9.9

2. Tissue Specific Glucose Uptake

At 120 min a bolus of [¹⁴C]2DG was given. Plasma samples were collected for the subsequent 25 min. Tissues were collected at 145 min and analyzed for [¹⁴C]2DG-P.

Time	Glucose mg/dl	[¹⁴ C]2DG dpm	AUC dpm·min
122	136	4648	51587.5
125	142	2257	
130	139	2879	
135	119	1907	
145	130	1378	

$$\text{Integrated plasma } [^{14}\text{C}]2\text{DG SA (dpm/ug)} = \frac{\left[\frac{\text{AUC} \cdot 220}{100 \cdot 10} \right]}{\text{Glucose avg (mg/dl)} \cdot 0.01 \cdot \text{time}}$$

Where AUC is the area under [¹⁴C]2DG curve period. When multiplied by 220 and divided by 1000 it will have units of (dpm/ul)*min. AUC is determined by trapezoidal rule. Time is the duration the AUC is measured. For example samples taken between 122 and 145 min would be a period of 23 min.

The rate of tissue glucose uptake (R_g)

$$R_g = \frac{\left[\frac{[^{14}\text{C}]2\text{DGP in tissue}}{\text{Tissue weight}} \right]}{\text{Plasma } [^{14}\text{C}]2\text{DGSA} \cdot \text{time}} = \text{ug glucose} \cdot \text{mg tissue}^{-1} \cdot \text{min}^{-1}$$

Multiply by 555 to convert to $\mu\text{mol glucose} \cdot 100 \text{ mg tissue}^{-1} \cdot \text{min}^{-1}$

Tissue	Weight mg	Volume of Base μl	Volume of Acid μl	A dpm	B dpm	A-B dpm	Tissue dpm	Tissue dpm/mg tissue	Plasma [¹⁴ C] 2DG SA dpm/ug	Rg $\mu\text{g/min/mg tissue}$
Soleus	22	16		817	321	496	6028	274	370	0.032
Gastro	61	16		2448	638	1810	21998	361	370	0.042
Vastus L.	63	16	45	3860	842	3018	37983	603	370	0.071
Diaphragm	60	16	30	5911	1620	4291	53387	890	370	0.104
Heart	63	16		12674	2140	10534	128026	2032	370	0.239
Adipose	55	16		330	105	225	2735	50	370	0.006
Brain	65	16	41	3498	559	2939	36876	567	370	0.067

Total amount of radioactivity in the homogenized tissue: Tissue (dpm)

$$\text{Tissue(dpm)} = \left[\frac{\text{measured (dpm)} \cdot (1250 + V_a + V_b)}{125} \right] \cdot \frac{1500}{1250}$$

Where V_a is volume (μl) of acid needed to neutralize PCA extract
 V_b is volume (μl) of base needed to neutralize PCA extract

Equipment, Tools and Reagents*

*The list below is only of items that are not easy to locate or where experience has indicated that a specific vendor is preferred.

Tracers

[3-³H]-Glucose. Perkin Elmer. NET331C

2-[1,2-³H]-deoxy-glucose. Perkin Elmer. NET549

2-[1-¹⁴C]-deoxy-glucose. Perkin Elmer. NEC 495 or **2-[¹⁴C]-deoxyglucose** NEC 720A

Surgical Tools

Forceps, Dumont 7b curved shank. Fine Science Tools # 11270-20

Hemostats. Fine Science Tools #13010-12

Micro-serrefines. Fine Science Tools # 18055-03 (straight); 18055-05 (curved)

Needle holder. Fine Science Tools # 12060-01

Spring scissors. Fine Science Tools # 15003-08

Scissors, surgical. Fine Science Tools #14028-10

Forceps. Roboz # RS-5136. RS-5110. RS-5111

Cautery. Cardinal Health # 65410-010

Miscellaneous Equipment

Heating pad. Harvard Apparatus # 72-0494

Glue, silicone. Ace Hardware (aquarium glue)

File, general purpose X slim taper file. Ace Hardware

Swivel, dual channel. Instech Solomon # 375/D/22QM

Connector, 3-way. Ziggy's Tubes and Wires # HSCY-25

Connector, 4-way. Ziggy's Tubes and Wires # HSC4-25

Blunt needle with luer hub. Ziggy's Tubes and Wires # LHN-E011041 25ga x 0.5"

Wire, stainless steel. Ziggy's Tubes and Wires # W020304V-1

Clamp, extension. Fisher Scientific # 05-769-7Q

Connector, hook. Fisher Scientific # 14-666-18Q

Stand, support. Fisher Scientific # 14-670A

Pump, single channel infusion. Harvard Apparatus # PY8 70-2208

Tubing

Stainless steel, 25ga. Ziggy's Tubes and Wires # 25R304-36

Microrenathane. Braintree Scientific # MRE-033

Silastic tubing 0.012"i.d. Fisher Scientific # 11-189-14

Silastic tubing 0.020"i.d. Fisher Scientific # 11-189-15A

Silastic tubing 0.058"i.d. Fisher Scientific # 11-189-15E

Polyethylene tubing (PE-10). Fisher Scientific # 14-170-12P

Polyethylene tubing (PE-20). Instech Solomon # BPE-T20

Miscellaneous Chemicals

0.3 N Ba(OH)₂. Sigma Aldrich # B4059

0.3 N ZnSO₄. Sigma Aldrich # Z2876

Urokinase (Abbott Labs # NDC 0074-6109-05)

Alteplase Cathflo® Activase® (# NDC 50242-041-64)