IV. Assay for Conversion of U-\(^{14}\)C-Glucose, \(^3\)H-Glycerol or \(^{14}\)C-pyruvate to Glyceride-Glycerol and Fatty Acids in Isolated Fat Cells

Principal Investigator: Michael P. Czech

This assay measures the flux of glucose, glycerol or pyruvate, added to isolated fat cells as radio-labeled substrates, into the glyceride moiety of triglyceride. Thus these assays can be used to estimate rates of esterification of fatty acyl CoA with glycerol 3 P derived from each of these substrates independently or in combination. Glycerol 3 P is formed from each of these substrates in fat cells, and is then converted into the glyceride portion of triglyceride in the esterification reaction (Glycerol 3 P acyltransferase).

Note: This protocol contains dangerous chemicals that can cause burns to exposed skin; take all precautions to prevent burns by wearing a lab coat, extra gloves, and eye/face protection. Make sure to use the fume hood properly. Keep away from open flames; some of the chemicals are highly flammable. Remember that \(^{14}\)C is used in this experiment and therefore materials in contact with the radioisotope should be disposed of properly.

Part A. Glucose, Pyruvate or Glycerol Conversion to Glyceride-Glycerol

1. ***Resuspend fat cells from about 1.0 g fat in 1.5ml-2mls KRH+2.5%BSA. (Optional: Let the cells rest for 20min in KRH buffer with BSA and 2mM pyruvate before adding them to the tubes listed below.)

2. \(^{14}\)C-Glucose Conversion to Glyceride Glycerol
   Label 15ml snap-cap polypropylene tubes. Dilute insulin from a stock of 2mg/ml, 1:3.3 in 3mM HCl making it a 100uM working stock. Add 0.9ml of KRH +2.5%BSA pH 7.4
to each tube. Add the other reagents below to the appropriate tubes. For each group of animals the following conditions (or others) can be tested:

- **Tubes 1,2,3 (NC)** - 10ul of a 1mM cytochalasin B (glucose transport inhibitor) solution to make the final concentration 10uM cytochalasin B
- **Tubes 4,5,6 (Control)** - 10ul of 3mM HCl
- **Tubes 7,8,9 (Insulin)** - 10ul of a 100mM insulin (made in 3mM HCl)

3H-Glycerol or 14C-Pyruvate Conversion to Glyceride Glycerol

Label 15ml snap-cap polypropylene tubes. Add 0.9ml of KRH +2.5% BSA pH 7.4 to each tube. Add other reagents (example conditions below) to the appropriate tubes.

- **Tubes 1,2,3 (glucose+insulin)** - 5ul of unlabeled glucose (at 1M working stock) + 10ul of 100mM insulin
- **Tubes 4,5,6 (No glucose +insulin)** - 5ul of PBS + 10ul of 100mM insulin
- **Tubes 7,8,9 (No glucose, no insulin)** - 5ul PBS + 10ul of 3mM HCl

3. Add 0.1ml of cells to all of the tubes, which contain the 0.9ml of KRH (plus additions as desired).

4. For measuring glucose conversion to glyceride glycerol add 50ul of a solution containing 20mM unlabelled glucose plus U-14C-D-glucose at 50uCi/ml.

For 32 tubes:

- 160ul unlabeled glucose (20mM working stock)
- 400ul 14C-Glucose (assuming a 200uCi/ml stock to give 80uCi total)
- 1040ul PBS
- 1600ul total

For measuring pyruvate conversion to glyceride glycerol, add 50ul of a solution containing

- 150 ul 14C-pyruvate
- 1350 ul PBS
- 1500 ul total
For measuring glycerol conversion to glyceride glycerol, add 50ul of a solution containing 20mM glucose with 1-\textsuperscript{3}H-glycerol at 50uCi/ml.

For 30 tubes:

150ul \textsuperscript{3}H-Glycerol (assuming a 0.5mCi/ml to give 75uCi total)
1350ul PBS
1500ul total

5. Incubate the cells at 37°C with shaking for 60 minutes (or more if desired).

6. ** At this point everything should be done in a fume hood. After 60 minutes add 5ml of Doles Extraction mixture cap and shake the tube, then let it stand for 15 min. Work with 9 tubes at one time(others capped) to avoid evaporation.

7. Add 3ml H\textsubscript{2}O and 3ml Hexane, and allow the phases to separate. Add the water and hexane to 9 tubes at one time only and cap them immediately (hexane evaporates very quickly).

8. **Remove the lower phase by aspirating** and wash the upper phase by adding 3ml of water and allowing the phases to separate. The lower solution contains water and 14C so should be poured down the sink in the “hot” room.

9. **Take 1ml of upper hexane phase** and put in glass scintillation vials marked “A” for part A and start evaporating.

10. Evaporate the hexane by low heating on heating plate(no open flames!!). Place the vials on a metal plate on top of a heating block in a fume hood.

11. Add 4mls of scintillation fluid and and measure radioactivity.
Part B. Conversion of Substrates (e.g., Glucose) to Free Fatty Acids

1. Take 1ml of upper phase from step 8 of Part A. and add to a 15 ml glass vial.

2. Evaporate the hexane by heating. Place the vials on a metal plate on top of a heating block in fume hood.

3. Add 1ml of ethanolic KOH (use a glass dispenser), (20 mls of 95% ethanol + 1ml of saturated potassium hydroxide) and heat at 80°C for 1 hour, capped.

4. Remove the tubes from the 80°C water bath and add 2ml of water per tube.

5. Add one drop of concentrated indicator (Nile Blue A) per tube.

6. Adjust the pH of the solution by using Nile Blue A to a neutral endpoint by viewing. Add 150ul of 6N (3M H₂SO₄) sulfuric acid until a blue endpoint and mix by vortexing after each addition of acid. ** Be careful to cap the tube before vortexing, KOH and sulfuric acid can cause severe burns, and ¹⁴C could contaminate objects close by. This indicator is a pink color at a pH greater than 7.0 and a blue color at pH 7.0 or less.

7. Add 4ml of hexane per tube, cap and shake vigorously for 30 seconds.

8. Allow phases to separate.

9. Remove 3mls and place in a scintillation vial.

10. Evaporate the solution as described in #2 of part B.

11. Add 4 ml of scintillation fluid and count.
To the remaining solution in the tubes from part B #1: Keep in the chemical fume hood with the caps off allowing the hexane to evaporate. When all hexane has evaporated, the liquid can be poured down the hot sink and the vials dumped in the appropriate containers. To the remainder of solution in tubes from Part B #9, put on a heating block and evaporate all liquid. These vials can be dumped in the scintillation vial container for $^{14}$C.

**Modified Doles Extraction Mixture as per Fain (100mls) 5mls/tube**

80mls isopropyl alcohol  
20mls hexane  
2mls 1 N $H_2$SO$_4$ (0.5M $H_2$SO$_4$)

**Saturated Potassium Hydroxide**

Add potassium hydroxide pellets to a 1-liter plastic beaker with approx. 500mls of water. Dissolve by stirring with a stir bar. Add more potassium hydroxide until the pellets no longer dissolve in the water. Pour the potassium hydroxide solution into a fresh bottle and let it cool.

**Ethanolic KOH**

Add 20mls of 95% EtOH to a glass jar, add 1ml of saturated potassium hydroxide to it jar, mix well.

**Nile Blue A Indicator Stock**

Mix powder with ethanol for a concentrated solution. Approximate concentration is 1mg/ml.