INTRODUCTION

Unlike strong acids, weak acids do not fully dissociate in solution. Instead they form the following equilibrium determined by the pKa of the acid and the pH of the solution:

\[ \text{HA} \text{(aq)} \leftrightarrow \text{H}^+ \text{(aq)} + \text{A}^- \text{(aq)} \]

The undissociated HA (aq) form is membrane permeable and can diffuse into the cytoplasm of the cell. Once dissociated, the anion A^- (aq) is membrane impermeable. Therefore the distribution of the weak acid is determined by the external pH (pH\text{e}) and the pKa of the weak acid. Benzoic acid has a pKa of 4.2.

This assay has been designed to measure the transmembrane pH gradient and thus pH\text{i} since the external pH is known. The magnitude of the pH gradient is calculated from the distribution of 14C-labelled benzoic acid and from knowledge of the external pH (pH\text{e}), the pH\text{i} can be calculated using the following equations:

\[ \Delta \text{pH} = \log \left( \frac{[\text{A}^-]}{[\text{A}^-]_0} \right) \left( 1 + 10^{pK_pH_0} \right)^{-10^{pK_pH_0}} \]

\[ \text{pH}_i = \text{pH}_0 + \Delta \text{pH} \]

The protocol for measuring the intracellular pH of \textit{E. coli} cells using C14-labelled benzoic acid is described below.

MATERIALS

MEDIA

- \textit{K}_{0.2} \text{ minimal medium:}
  - \textit{K}_0 \text{ buffer per litre dH}_2\text{O:}
    - Na_2HPO_4,12H_2O 16.47 g
- NaH$_2$PO$_4$.2H$_2$O 3.58 g
- (NH$_4$)$_2$SO$_4$ 1.05 g

- 100 mM KCl (X200 stock) added to K$_0$ buffer to achieve a final [K$^+$] 0.2 mM.

- Growth supplements:

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Stock Conc.</th>
<th>Dilution</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>20 % (w/v)</td>
<td>100X</td>
<td>0.2 % (w/v)</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>0.01 % (w/v)</td>
<td>100X</td>
<td>0.0001 % (w/v)</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>40 mM</td>
<td>100X</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$.FeSO$_4$.6H$_2$O</td>
<td>6 mM$^a$</td>
<td>1000X</td>
<td>6 µM</td>
</tr>
</tbody>
</table>

$^a$Stock solution made up in 0.1 M HCl

All growth supplements are autoclaved prior to use and added to K$_{0.2}$ buffer to make K$_{0.2}$ media.

REAGENTS
- Na$_2$HPO$_4$.12H$_2$O
- NaH$_2$PO$_4$.2H$_2$O
- (NH$_4$)$_2$SO$_4$
- 100 mM KCl
- 20 % D-Glucose
- 0.01 % Thiamine.HCl
- 40 mM MgSO$_4$.7H$_2$O
- 6 mM (NH$_4$)$_2$SO$_4$.FeSO$_4$.6H$_2$O
- 4.5 µM 14C-Benzoiac Acid (0.1 µCi.ml$^{-1}$)
- 3H-Water (1 µCi.ml$^{-1}$)
- 56 mM Methylglyoxal
- Scintillation fluid

REAGENT SET-UP

EQUIPMENT
- Incubator (New Brunswick Scientific Co. Ltd. G-25 Incubator Shaker set to 200 rpm)
- Circulating Water bath at 37°C
- Thermally insulated pots
- Micro-centrifuge (Jouan A14 ultracentrifuge, 14000 rpm)
- Filtration unit
- Vacuum pump
- Aspirator
- Jouan MR22i centrifuge, SWM180.5 rotor
- Scintillation counter
**PROCEDURE**

**Experimental preparation**

Check radioactivity monitoring form for work area to ensure bench is safe to use. Swab work area before commencing the experiment. Calculate volumes of radioactive stocks required for experiment and collect **only the required volume** from the radioactive suite. Radioactive substances should be transported in a sealed box. (For this experiment this can be done while the cells are growing).

**Sample preparation**

1. Inoculate a falcon tube containing 10 ml K\textsubscript{0.2} medium with a single colony
2. Grow culture overnight at 37°C, 200 rpm.
3. Dilute culture 1/8 into 60 ml K\textsubscript{0.2} medium prewarmed to 37 °C (OD\textsubscript{650} ~ 0.1) and grow at 37 °C, 200 rpm until OD\textsubscript{650} ~0.8.
4. Harvest 33 ml cells onto a nitrocellulose membrane by filtration.
5. Wash cells with 10 ml K\textsubscript{0.2} buffer prewarmed to 37 °C
6. Resuspend cells in 33 ml K\textsubscript{0.2} buffer + 0.2% Glucose prewarmed to 37 °C.
7. Transfer 16 ml cell suspension into two thermally insulated glass pots and stir continuously = **Time 0** (NB: have ~30 ml cells left over, filter, wash and resuspend cells as above, harvest by centrifugation at 4000 rpm for 10 min at room temperature. Discard supernatant and resuspend pellet in 1/5 volume, 6 ml, K\textsubscript{0.2} buffer + 0.2 % Glucose – this gives cells of OD \textsubscript{650} ~ 0.4 and this is important for the end controls so keep)
8. After 5 min take 1 ml cell sample for OD\textsubscript{650} measurement = **Time 5 min**
9. Add 15 µl 3H-Water (final activity 1 µCi.ml\textsuperscript{-1}) and 15 µl 14C-Benzoic Acid (f/c 4.5 µM / 0.1 µCi.ml\textsuperscript{-1})
10. Incubate for 5 min to allow weak acid to equilibrate across the membrane = **Time 10 min**

11. **Sampling:**

   **Take samples at 10, 12, 14, 15.25, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30 min (= 14 samples total)**

   Remove 1ml samples into eppendorfs.
   Spin 14,000 rpm x 20 sec.
   Transfer 100 µl supernatant into scintillation vial.
   Aspirate remaining supernatant. The pellet can be left on bench until all time points have been collected and sampling is finished (be sure to cap tubes).
   For each time point there are 2 samples: \textbf{S}, supernatant (in scintillation vial) and \textbf{P} pellet (in eppendorf).

   \textbf{P, pellet sample:} Resuspend pellet in 200 µl K\textsubscript{0.2} buffer by vortexing. Transfer 200 µl into scintillation vial, add a further 100 µl K0.2 buffer to the scintillation vial.
Total volume in scintillation vial = 300 µl.

**S, supernatant sample:** Add 200 µl of cell suspension at an OD_{650} ~ 4 (created at step 7) into the scintillation vials for the supernatant samples.

Total volume in scintillation vial = 300 µl

**Controls**

1. **Blank** (no radioactivity): 200 µl OD_{650} 4 cell suspension + 100 µl K_{0.2} buffer + 0.2% Glucose.
2. **14C-Benzoinic acid standard:** 200 µl OD_{650} 4 cell suspension + 100 µl K_{0.2} buffer + 0.2% Glucose + 1 µl C14-Benzoinic acid (f/c 4.5 µM / 0.1 µCi.ml^(-1)).
3. **3H-Water standard:** 200 µl OD_{650} 4 cell suspension + 100 µl K_{0.2} buffer + 0.2% Glucose + 1 µl 3H-Water (1 µCi.ml^(-1)).

**12. Scintillation Counting**

Add 3 ml Scintillation fluid to all scintillation vials (contamination checks + experimental samples)

Record the scintillation counts using a program for the double label (3H/14C, #7)

(NB: if scintillation counting is done overnight ensure all paper work is completed first thing next morning).

**13. Finishing the Experiment**

Swab work area following completion of the experiment to check for contamination Complete IsoInventory entry and all required paper records.

**14. Calculating pH_i from the raw data**

The following values are needed to calculate the pH_i:

- For each sample the 3H and 14C values of the supernatant (S) and pellet (P) from the scintillation counter
- The OD_{650} of the cell suspension (measured at t=5min) so the cytoplasmic volume (V) of the cells can be calculated. For this take an OD_{650} 1 to be 1 mg dry weight cells and 1 mg dry weight cells to have a cytoplasmic volume of 1.6 µl (Stock _et al._, 1977).
- The blank values (B) for 3H and 14C from the scintillation counter
- The 3H/14C channel overlap ratio determined by the 3H and 14C counts from the 14C-Benzoinic acid standard. Typically the 14C overlap in the 3H channel is so small as to be negligible in the pH_i calculation but the 3H-water standard is carried out to check this in every experiment.
- The pH_o (pH 7)
- The pKa of benzoic acid (4.2)

To calculate the pH_i:

1. Correct the 3H and 14C supernatant and pellet values for the blank values.
2. Correct the 14C values for the supernatant and pellet for the 3H overlap.
3. Calculate the contaminating 14C in the pellet; Divide the C14 value by the 3H value for the supernatant sample and multiply by the 3H value for the pellet.
4. Calculate the intracellular 14C by subtracting the above contamination value from the 14C value for the pellet.
5. Calculate the accumulation ratio for benzoic acid by dividing the intracellular 14C by the supernatant 14C value. Correct the accumulation value for the cytoplasmic volume of the pellet.
6. Using the Accumulation ratio (A), the pK of benzoic acid, and the external pH (pH₀) calculate pHᵢ as follows:

\[ \Delta pH = \log \left( 1 + 10^{pK-pH₀}\right) - 10^{pK-pH₀} \]

\[ pHᵢ = pH₀ + \Delta pH \]

References


