Amino Acid Write Up

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1. Plot [14C]-isoleucine (counts min-1) against time for each experiment, including error bars for ± SEM.

See Figure 2. attached.

2. For the 15 min time point of each experiment calculate and tabulate the following:

i) The internal concentration of isoleucine. Determine using an appropriate test whether the internal concentration of isoleucine is statistically different between appropriate experiments.

Full results are attached on Figure 3., the following are example questions worked through to show method. To calculate the internal concentration of [14C]-isoleucine within the filter examined we need to:

(1) Calculate the number of moles of [14C]-isoleucine needed to give a reading of xCPM.
(2) Calculate the number of moles required to give the readings we observed in each experiment (nN).
(3) Calculate the internal cell volume.
(4) Divide nN by the internal cell volume.

1. A 10µl sample of 0.26mM [14C]-isoleucine registered 576 CPM.

We'll initially subtract the background radiation, approximately 10 CPM ; 576-10 = 566 CPM

We'll then convert the mM and µl figures into M and l ; 0.26/1000 = 0.00026M ; 10/1,000,000 = 0.00001l

We'll then calculate the number of moles in the reference sample; 0.00026M * 0.00001l = 0.0000000026 moles

Hence, we require 0.0000000026 moles of [14C]-isoleucine to deliver a CPM reading of 566 (minus background radiation).

From this we can calculate the number of moles required to give a reading of x CPM.

Moles required for x CPM = $0.0000000026/(566/x)$

2.

e.g Moles required for 736 CPM (experiment 1, cpm1, at t=15, -background radiation)

Moles required for 736 CPM = $0.0000000026/(566/736)$ = 0.000000003381 moles

3. We now need to calculate the internal cell volume of the bacteria on the filter - While the cells were resuspended before the experiment took place to 1 mg dried weight / ml-1 - The addition of [14C]-isoleucine occurred AFTER resuspension. This will change the mg dried weight / ml-1 of the flask, so this needs to be recalculated.

The cells were resuspended to 1 mg dried weight / ml-1, therefore in the 10ml added to the conical flask each ml will contain 1 mg dried weight / ml -1.

250µl of [14C]-isoleucine was then added, increasing the overall volume of the liquid in the flask, decreasing the mg dried weight / ml -1.

1ml was extracted at t=1, t=5, t=10 and t=15.

1000/10250 = 0.09756098
0.09756098*10 = 0.9756098
0.9756098*1.55µl = 1.512µl

For experiments 2, and 4-6, 0.25ml of 21mM glucose was also added. The addition of this requires us to recalculate internal cell volume for these experiments also.

1000/10500 = 0.0952381
0.0952381*10 = 0.952381
0.952381*1.55µl = 1.47619µl

For experiment 3, 5µl of CCCP was also added, as well as 0.25ml of 21mM glucose. The addition of this requires us to recalculate internal cell volume for this experiment.

1000/10505 = 0.09519277
0.09519277 * 10 = 0.9519277
0.9519277*1.55µl = 1.4754879µl

4. We can now calculate the concentration of [14C]-isoleucine on the filter:

e.g Concentration of [14C]-isoleucine within experiment 1, cpm1, at t=15, -background radiation

0.000000003381 moles / 0.000001512l = 0.002236 M

0.002236 * 1000 = 2.236mM

A series of two tailed t.tests, assuming equal variances were performed on samples to see if there were statistically significant difference between experiments, with and without uncouplers present.

All three uncouplers; CCCP, valinomycin and nigericin, are all significantly different to the experiment just performed with glucose ($\rho = 0.00000025905$; $\rho = 0.002236$; $\rho = 0.0000007601$ respectively). In comparison to each other, the difference between uncouplers in isolation are (CCCP:val $\rho = 0.0009410$; CCCP:nig $\rho = 0.00002315$; val:nig $\rho = 0.007665$ respectively).

In fact the null hypothesis was rejected for every experiment in relation to all others, except one between +Glucose, +CCCP and +Glucose, +Nigericin, +Valinomycin. This t. test did not reject the null hypothesis ($\rho = 0.2191$)and implied there's no significant difference between the two effects of CCCP; and nigericin and valinomycin in combination.

ii) The calculated external concentration of isoleucine.

TO MARKER: When running these calculations I kept getting negative results (which have been included), the intracellular concentration of isoleucine for all cells seems to be greater than the isoleucine added at the beginning - I'm honestly not sure where I'm going wrong on this, and would really appreciate feedback on where I went wrong! The external concentration of isoleucine at t=15mins can be calculated by:

(1) Calculate the total number of moles added to the solution at t=0
(2) Calculate the number of moles uptaken into all cells at t=1
(3) Calculate number of moles not in cells at t=1
(4) Calculate concentration of liquid at t=1
(5) Calculate the number of non-intracellular moles removed at t=1
(6) Calculate the total number of cells removed at t=1
(7) Repeat process for t=5 and t=10
(8) Calculate number of moles within cells at t=15
(9) Add number of moles within cells at t=15 to number of moles removed at t=1, t=5 and t=10
(10) Calculate the number of moles remaining in solution
(11) Calculate the concentration in solution at t=15

1. The total number of moles added to the solution can be calculated as follows:

250ul * 0.26mM = 0.00025l * 0.00026M = 0.000000065 moles were added ie total number of moles in the solution is 0.000000065

2. Number of moles uptaken into all cells at t=1; can be calculated by using the number of moles calculated from the CPM in the sample, divided by the fraction of cells that are in the sample examined.

e.g Experiment 1, cpm1, t=1; t=5; t=10, - background radiation

Moles required for x CPM = $0.0000000026/(566/x)$

t=1

Moles required for 672 CPM = $0.0000000026/(566/672)$ = 0.000000003087 moles

0.09756098/1 of the cells are on the filter [see Q2)i)3)]

0.000000003087 / 0.09756098 = 0.00000003164174858 moles

3. Calculate no. of moles not in cells at t=1

This is simply the total number of moles added at t=0, minus the moles present in cells at t=1

0.000000065 - 0.00000003164174858 = 0.0000000333582514 moles not in cells

4. Calculate the concentration in solution at t=1

This can be calculated by the number of moles not present in cells, divided by the (total volume - internal cell volume)

The volume can be calculated as 10250µl - 15.5µl = 10234.5µl = 0.01023451

0.00000003335825142 moles / 0.01023451 = 0.000003259391 M

5. Calculate the number of moles removed at t=1, in the 1ml, that isn't present within cells

We can work this out by multiplying the concentration by the volume of liquid removed (1ml - 1.512µl)

1ml - 1.512µl = 0.998488 = 0.0009984881

0.000003259392391 M * 0.0009984881 = 0.000000003254464 moles removed not in cells

6. Calculate number of moles removed at t=1

We calculate this by adding the number of moles removed that weren't in cells from the number of moles removed that were in cells

0.00000003254464 19 moles not in cells, but in 1ml extraction + 0.000000003087 moles in extracted cells = 0.00000000634146419 moles removed at t=1

7. We repeat the process for t=5, and t=10, results on the attached sheet, but account for the moles and cells already removed.

Full worked example for t=5 follows (for experiment 1, cpm1):

832 CPM reported = 822 CPM (- background radiation)

0.0000000026/(566/822) = 0.000000003776 moles required for CPM

Total moles in all cells (accounting for cells removed in t=1) = 0.000000003776/(0.09756098/(1-0.09756098)) = 0.00000003492799826 moles

Total moles not in cells at t=5 = 0.000000065 (total added at t=0) - 0.00000003492799826 (total moles in all remaining cells at t=5) - 0.00000000634146419 (moles removed in t=1) = 0.00000002373053755 moles

External concentration at t=5 = 0.00000002373053755 (total moles not in cells) / ((0.0102345-0.000998488) (Total liquid in flask - liquid removed at t=1) - (0.0000155-0.000001512) (Total internal cell volume - cell volume removed in t=1)) = 0.000002573246128 M

Moles present in 1ml extraction, not in cells = 0.000002573246128 (molarity of liquid in flask at t=5) * 0.000998488 (Liquid in 1ml extraction - internal cell volume in 1ml extraction) = 0.00000000256935538 moles

Total number of moles removed at t=5 = 0.00000000256935538 (Moles in liquid in 1ml extraction) + 0.000000003776 (Moles in cell volume in 1ml extraction) = 0.00000000634535538 moles

Full worked example for t=10 follows (for experiment 1, cpm1):

851 CPM reported = 841 CPM (- background radiation)

0.0000000026/(566/841) = 0.000000003863 moles required for CPM

Total moles in all cells (accounting for cells removed in t=1 and t=5) = 0.000000003863/(0.09756098/(1-(2*(0.09756098))) = 0.00000003186974822 moles

Total moles not in cells at t=10 = 0.000000065 (total added at t=0) - 0.00000003186974822 (total moles in all remaining cells at t=10) - 0.00000000634535538 (moles removed in t=5) - 0.00000000634146419 (moles removed in t=1) = 0.00000002044343221 moles

External concentration at t=10 = 0.00000002044343221 (total moles not in cells) / ((0.0102345-(2*0.000998488)) (Total liquid in flask - liquid removed at t=1 and t=5) - (0.0000155-(2*0.000001512)) (Total internal cell volume - cell volume removed in t=1 and t=5)) = 0.000002485509168 M

Moles present in 1ml extraction, not in cells = 0.000002573246128 (molarity of liquid in flask at t=10) * 0.000998488 (Liquid in 1ml extraction - internal cell volume in 1ml extraction) = 0.000000002481751078 moles

Total number of moles removed at t=10 = 0.000000002481751078 (Moles in liquid in 1ml extraction) + 0.000000003863 (Moles in cell volume in 1ml extraction) = 0.000000006344751078 moles

8. We then calculate the number of moles present within cells at t=15 (using the same method as in step 1 & 2), except account for the cells already removed:

= 736/ (0.09756098/((1-0.09756098*3))) = 0.00000002451 moles

9. We then add the number of moles present within cells at t=15, to the moles already removed at t=1, t=5 and t=10.

0.00000002451165922 (moles present in cells at t=15) + 0.00000000634146419 (moles removed at t=1) + 0.00000000634535538 (moles removed at t=5) + 0.000000006344751078 (moles removed at t=10) = 0.00000004354322987 moles

10. We then calculate the number of moles remaining in solution

0.000000065 - 0.00000004354322987 = 0.000000021456770 moles

11. We can then calculate the concentration at t=15

Remaining volume inside cells = 10250µl - 3ml - internal volume of remaining cells) = 10250µl - 3000µl - (15.5-3(1.512))µl =7239.036µl

7239.036µl = 0.007239036l

0.000000021456770 moles / 0.007239036l = 0.000002964036943 M

This are the calculations carried out for each experiment. The results are on the attached Figure. 3

iii) The factor by which [14C]-isoleucine has been concentrated within the cells. The factor of concentration within the cell at t=15 can be calculated from the concentration within the cell at t=15, and the concentration outside the cell at t=0.

e.g experiment 1, cpm1, t=1; t=5; t=10, - background radiation

2.236mM within the cell at t=15

0.000000065 moles / 0.010248451 = 0.000006342422513M concentration in flask at t=0

0.006342mM outside the cells at t=0

2.236/0.006342 = 352.56

Hence it has been concentrated within the cell by a factor of 352.56 for experiment 1, cpm1. The results for all experiments are on the attached Figure. 3.

Included in the spreadsheet are 3 experimental datasets (PROBLEM DATA 1, 2, 3) that suggests a problem has occurred in the execution of the experiment. In each case provide a reasonable explanation of what MIGHT have gone wrong with the experiment.

Problem data 1 has incredibly an incredibly low CPM reading. The calculated [14C-isoleucine] of this sample is very similar to the homogeneous concentration of [14C]-isoleucine within the conical flask at t=0 (6.65885E-10M vs 6.6625E-10M). There was, effectively, no concentration of radioactivity within the sample. This leads me to conclude the bacteria were a) not present in the sample examined on (or present in minimal concentration), b) were present, but had all died before the experiment took place or c) were somehow all lost before the reading was taken.

I believe the most likely error is that bacteria not being present in the conical flash the experiment was performed on is the most likely error. This may have occurred during washing and resuspension using photospectrometry, it's possible the cuvette used to measure the $OD_{6502.2}$ was dirty/scratched and so gave a much higher reading, leading to all the bacteria within the cuvette to effectively be washed out by the phosphate buffer. And so the glucose and nigericin was added to a phosphate buffer, resulting in no concentration of [14C]-isoleucine.

(Assuming the the magnitude of the experiment 1 results aren't erroneous (despite statically significant different to the other 3 counts of experiment 1 results when a t.test is applied; $\rho = 0.0431$)), The issues with these results is there's no clear increase in CPM when glucose is added in experiment 2. I would assume the error occurred in adding the glucose in experiment 2. I would suggest the error occurred was that instead of adding 0.25ml, the experimenter read the instructions as 0.25ul, or had miscalibrated their pipette, resulting in the glucose added being a factor of 10x or 100x smaller than the glucose that should have been added, leading to no effective change in CPM.

The data for problem data 3 is the most chaotic by far. The cpms at the 10 minute mark would indicate nearly 10% of the [14C-isoleucine] is within the bacteria sample. This is also particularly unusual given there is no real net change between the 1 minute mark and the 5 minute mark, when in the standard data the only real increase in [[14C]-isoleucine] occurs between the 1 minute reading and 5 minute reading. For that reason, and the enormous variety in these readings, I believe the error is either a) with the equipment, b) how the reading was taken or c) a contaminant was somehow introduced between minute 10 and 15.

If it's a contaminant, it's possible the 1ml extractions at the 10 minute mark and 15 minute mark were using the same pipette tip used to add the [14C]-isoleucine, however I think this explanation is unlikely (Why would the experimenter reuse a tip, after using new ones for the past two 1ml extractions?). Contamination of the filters or the 1ml extraction seems most likely, however I find it difficult to find a route this could have occurred through.

It's also possible the readings at the 10 minute mark and the 15 minute mark were taken near the [14C]-isoleucine reserve, and this threw off the count, or the liquid scintillation counter or some of the equipment used in the process is faulty.

Briefly, (600 words maximum) discuss your data with respect to the mechanism of transport of isoleucine into Staphylococcus aureus*, explaining the mode of action of each of the uncouplers used in your discussion.*

The active (Δp dependant) uptake of isoleucine uptake into *Staphylococcus aureus* occurs by the action of a H+ coupled symport **?**. As $\Delta p = \Delta\Psi + \Delta pH$, any reduction in the electrical or chemical potential difference over the cytoplasmic membrane may result in a reduction of isoleucine uptake. The first two experiments performed (No Glucose and +Glucose) show the effect a fully functioning electron transport chain has on [14C]-isoleucine uptake. Under the presence of glucose (Exp2.), the bacterial cells are able to concentrate roughly 4x more [14C]-isoleucine into the cytoplasm (2.411mM : 8.226mM). The ionophores CCCP, valinomycin and nigiricin, used in experiments 3-6 (along with glucose) uncouple oxidative phosphorylation, by providing alternative routes for $\Delta\Psi$, ΔpH or both to equilibrate.

Carbonyl Cyanide *m*-chlorophenyl hydrazone (CCCP) is a lipid soluble weak acid, and protonophore that selectively increases the bacterial cytoplasmic membranes permeability to H+ ions **?**. It acts by diffusing into the cytoplasmic membrane in its HA form and releasing a H+ ion into the cytoplasm, A- is then free to diffuse through to the positively charged side of the membrane (at a voltage dependant rate) and adsorb another H+ ion **?**. As the protons now have an alternative route down their concentration gradient out of the cell, this vastly reduces the effectiveness of the electron transport chain to maintain Δp. As both ΔpH and $\Delta\Psi$ are reduced under the action of CCCP, the potential ΔG released from returning the [H+] to equilibrium is reduced, making the translocation less energetically favourable, resulting in reduced uptake of [14C]-isoleucine. The results are however, significantly different to the concentration of isoleucine within a cell starved of glucose ($\rho = 0.0009$), showing how even a damaged ETC is more effective than the inability to power one.

Valinomycin is an neutrally charged depsipeptide ionophore ,with high selectivity for K+ ions (over 10,000x favourability of K+ over Na+) **??**. The expected hypothetical result of addition of valinomycin will be a reduction in $\Delta\Psi$ over the cytoplasmic membrane. As an ionophore for K+ ions, it allows a route of equilibration for $\Delta\Psi$ as K+ can now move freely into the cytoplasm to counteract the $\Delta\Psi$ produced by the electron transport chain. This,

PROBLEM DATA 1

Experiment 5, +Glucose, + Nigericin

Time (mins)	cpm1
1	15
5	14
10	12
15	12

PROBLEM DATA 2

Experiment 1, No Glucose

Time (mins)	cpm1
1	936
5	888
10	916
15	896

Experiment 2, + Glucose

Time (mins)	cpm1
1	1001
5	986
10	931
15	895

PROBLEM DATA 3

Experiment 1, No Glucose

Time (mins)	cpm1
1	1125
5	1150
10	10384
15	4187

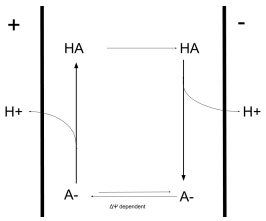

Figure 1. Action of CCCP within the cytoplasmic membrane

in turn, will reduce Δp, which will reduce the ability of *S. aureus* to uptake isoleucine. Compared to CCCP, which directly affects both ΔpH and $\Delta\Psi$, valinomycin's effect on Δp is significantly smaller ($\rho = 0.0009373$) - with concentrations of [14C]-isoleucine within cells treated with valinomycin being nearly twice as high as in cells treated with CCCP.

Nigericin is an ionophore with an overall structure similar to valinomycin **?**, and like valinomycin nigericin is an ionophore for K+, however when accepting the K+ ion, nigericin releases a H+ ion (allowing it to remain uncharged in an electroneutral exchange) **?**. The biochemical result of nigericin's action is an equalisation of both K+ and H+ concentrations between the cell and the periplasm. The net result is a reduction in ΔpH, as K+ is transported out of the cytoplasm, [H+] increases within the cytoplasm to reducing ΔpH over the cytoplasmic membrane.

When used in combination with valinomycin, the cell has a reduction of ΔpH AND $\Delta\Psi$. Nigericin equilibrates ΔpH, while valinomycin allows equilibration of $\Delta\Psi$ by providing an another route for K+ ions in and out of the cell. The joint effect vastly reduces Δp, in an overall effect similar to CCCP, which also reduced $\Delta\Psi$ and ΔpH. This can be seen by the t-test performed between +Glucose, +CCCP and +Glucose, +Nigericin, +Valinomycin, which showed no statistically significant difference between the effects of the two experiments on [14C]-isoleucine uptake ($\rho = 0.2146$).