BCH3346 – EXAM PRACTICE QUESTIONS 2014

ANSWERS

Lab 1: TRANSAMINASES

1) Assuming that the salt fractionation experiment (steps 10 to 21 of experiment 1) produces the following results:

% salt saturation	% GOT in pellet	% protein in pellet
50	2	15
55	10	40
60	60	62
65	92	76
70	95	85

Describe a protocol for a two-step purification of GOT by salt precipitation. Indicate the recovery and the purification factor (relative increase in specific activity) of GOT to be expected with that protocol.

- **Perform the first precipitation at 55 % salt saturation. Keep the supernatant and discard the pellet. Going to 60% salt saturation will remove an extra 22 % of protein for an extra 50% loss in enzyme.**

- **Perform a second precipitation of the previous supernatant by increasing salt up to 65 % saturation. Keep the pellet and discard the supernatant. Going to 60% salt will provide lower yield without an increase in purity.**

- **Recovery (yield) = 92 (% GOT in pellet from 2nd precipitation) – 10 (% GOT in pellet from 1st precipitation that was discarded) = 82 % of the GOT activity**

- **Specific activity in homogenate = 100% GOT/ 100% protein = 1.0**

- **Specific activity in purified sample (2nd pellet) = 82% GOT /(76-40)% protein = 2.28**

- **Purification factor: 2.3 / 1.0 = 2.3**

2) For the coupled assay of GPT with LDH, under condition of substrate saturation, the rate of NADH utilization should increase if: (Don't forget to justify your answers).

True	False	
(___)	(___)	The amount of LDH is increased.
(___)	(___)	The amount of NADH is increased.
(___)	(___)	The amount of alanine is increased.
(___)	(___)	The amount of GPT is increased.

1. FALSE:

- **LDH should be in excess so that the limiting step is the GPT reaction (if LDH is increased the rate of the LDH reaction shouldn't increase since in the coupled assay, the transaminase reaction is rate limiting)**

2. FALSE:

- **NADH should be at saturation level to make sure that the condition 1 is fulfilled (rate is limited by rate of pyruvate formation by TA reaction, so increasing NADH levels has no effect).**

3. FALSE:

- **substrates are used at saturation, thus, the increase in alanine concentration does not affect the rate of neither the first nor the second reaction. The assay is independent of the substrate concentration.**

4. TRUE:

- **The assay (rate of NADH conversion) is solely dependent on the amount of GPT present, since this is the rate limiting step.**

3. An unknown concentration of alanine was incubated with 0.3 mM α-ketoglutarate, 0.2 mM NADH, GPT (glutamate-pyruvate transaminase) and LDH (lactate dehydrogenase) until the mixture's absorbance at 340 nm stabilized at 0.622. (ε340 NADH = 6220 $M^{-1}cm^{-1}$.) Calculate the initial concentration of alanine and the final concentrations of alanine, α-ketoglutarate, glutamate, pyruvate, lactate and NADH.

- **Final concentration of NADH = $\frac{A}{\varepsilon * l} = \frac{0.622}{(6220\ M^{-1}cm^{-1}) * 1cm} = 1 \times 10^{-4} M$ = 0.1 mM**

- **Knowing initial concentration of NADH (0.2mM), we can calculate the decrease in NADH as follows:**

  NADH = 0.2mM – 0.1 mM = 0.1 mM

- **Since the conversion of pyruvate to lactate requires the oxidation of one NADH, we can determine the final concentration of lactate based on the amount of NADH oxidized, which we have just calculated above:**

  Final [Lactate] = 0.1 mM

- **Since there was still some NADH remaining in the reaction mixture, we can infer that the amount of pyruvate produced by the GPT reaction was entirely depleted (otherwise, more lactate would have been formed, leading to a further reduction in the concentration of NADH). Therefore:**

  Final [Pyruvate] = 0.0

- **The GPT reaction produces equal amounts of the products pyruvate and glutamate. Since we know that 0.1 mM of pyruvate was produced (because 0.1mM of NADH was oxidized in the LDH reaction), this has to mean that the final concentration of glutamate is also 0.1 mM. Therefore:**

  Final [Glutamate] = 0.1 mM

- **Since the final concentration of glutamate is 0.1 mM, this means that the intial concentration of alanine in the reaction is also 0.1 mM. Therefore:**

  Initial [Alanine] = 0.1 mM

- **Finally, 0.3 mM of alpha-ketoglutarate was present in the initial reaction, but we know that only 0.1 mM was required to produce 0.1 mM of the product glutamate, therefore:**

  Final [α-ketoglutarate] = 0.3 – 0.1 = 0.2 mM

- **Given that alpha-ketoglutarate was in excess and not entirely depleted, this means that the initial concentration of alanine was limiting, therefore:**

  Final [Alanine] = 0.0

Lab 2: MITOCHONDRIA

1) Complete the following table with the corresponding expected (theoretical) values for the incubation of mitochondria with the indicated substrates in the presence and absence of malonate. Explain your reasoning.

Table 1. Theoretical values

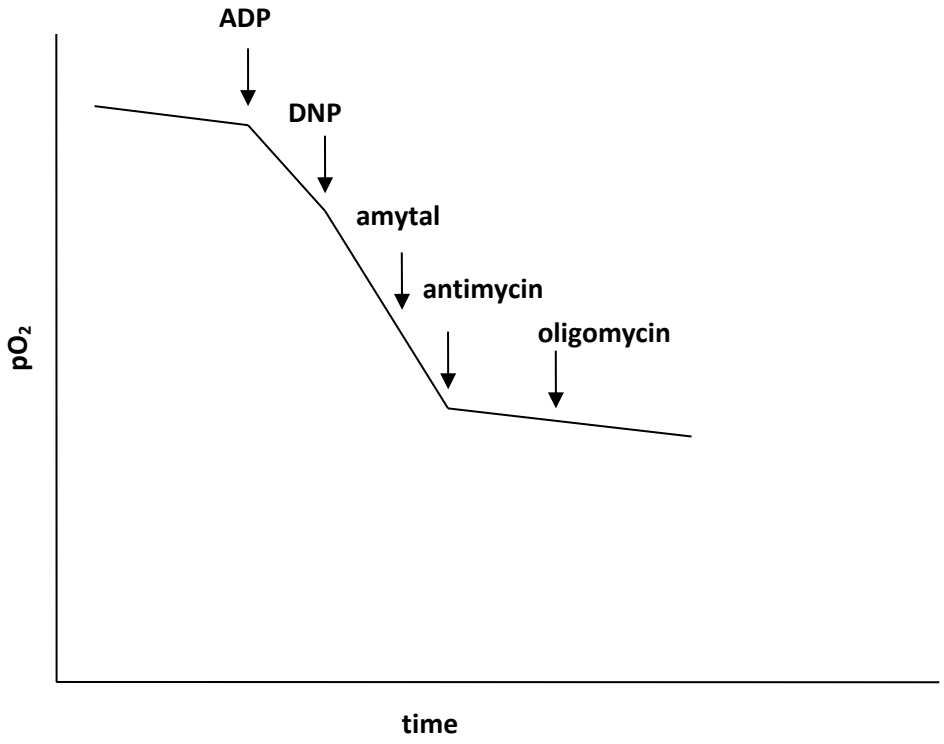
Substrate	Inhibitor	O_2/substrate	Pi/substrate	Pi/O
Hydroxybutyrate	----------	0.5	2.5	2.5
Hydroxybutyrate	Amytal	0	0	0
Succinate	----------	0.5	1.5	1.5
Succinate	Amytal	0.5	1.5	1.5
α-Ketoglutarate	----------	1.0	5.0	2.5
α-Ketoglutarate	Amytal	0.5	2.5	2.5

- **β-Hydroxybutyrate is oxidized by NAD+ to acetoacetate. The resulting NADH is oxidized in the electron-transport chain by one O (0.5 O_2) and produces NAD+ and H_2O along with the translocation of 10 protons. This proton gradient provides the energy for the phosphorylation of 2.5 ADP. The oxidation of NADH is inhibited by amytal at the level of Complex I. Therefore, the resulting NADH cannot be oxidized and no ATP is produced.**

- **Succinate is oxidized by FAD+ to $FADH_2$ and fumarate. The $FADH_2$ is oxidized by one O (0.5 O_2) to FAD+ and H_2O in the electron-transport chain resulting in the translocation of 6 protons that provide the energy for the phosphorylation of 1.5 ADP. The oxidation of $FADH_2$, which bypasses complex I and instead uses complex II, is not affected by amytal which inhibits only the activity of complex I.**

- **α-Ketoglutarate is oxidized by NAD+ to NADH and succinyl-CoA which upon conversion to succinate produces one GTP (equivalent to one ATP). Succinate is oxidized by FAD+ to fumarate and $FADH_2$. Amytal is an inhibitor of Complex I, therefore there will be no oxidation of NADH, reducing the proton gradient by 10 protons (energy for the phosphorylation of 2.5 ADP)**

2) Isolated rat liver mitochondria were resuspended in a buffer containing an excess of succinate; an oxygen electrode was placed in the suspension. The oxygen consumption was recorded. Draw the expected plot for oxygen consumption when ADP, DNP, amytal, antimycin, and oligomycin are added sequentially. Explain your answer.



- **ADP increases the rate of oxidation as the proton gradient is being used for phosphorylation, therefore we see a change in the slope which corresponds to an increase in the rate of oxygen consumption.**
- **DNP eliminates the proton gradient and, thus, removes any opposition to oxidation.**
- **Amytal acts at the level of Complex I and has no effect on the oxidation of succinate by Complex II, therefore the rate of oxygen consumption is unaffected (no change in slope)**
- **Antimycin inhibits Complex 2 and blocks the oxidation of succinate, which is why we see the change in the slope which corresponds to an almost complete stop in oxygen consumption.**
- **Olygomycin has no effect on oxidation since the system is uncoupled at this point and there is no phosphorylation (there is no proton gradient).**

3) You perform a first incubation of mitochondria with β-hydroxybutyrate, phosphate and a limiting amount of ADP and let it run longer than required for depletion of the ADP. You now perform a second incubation in similar conditions but in the presence of DNP, and a third one in the presence of oligomycin (but no DNP). Which incubation will show the highest consumption of oxygen and which one the highest consumption of phosphate? Explain.

- **The 2nd incubation will show the highest consumption of oxygen since there is no limitation by ADP (ie. because DNP is uncoupling the process of oxidative phosphorylation). The third incubation will show the lowest consumption, since in intact mitochondria, oligomycin will stop oxidation.**

- **The 1st incubation will show the highest consumption of phosphate because ADP will be phosphorylated. The amount of Pi used will be equal to the amount of ADP. The 2nd incubation will show the lowest amount of phosphate, since there is no phosphorylation. Oligomycin will inhibit phosphorylation, but not 100%, thus it will show a very low amount of Pi consumption.**

Lab 3: ADIPOSE TISSUE

1. When you incubate adipose tissue with (U-^{14}C) glucose, the addition of adrenaline to the incubation mixture will have the following effects: (Remember to justify your choices)

	True	False	
a-	(___)	(___)	the amount of metabolized glucose will decrease.
b-	(___)	(___)	the amount of radioactivity in the released CO_2 will increase.
c-	(___)	(___)	the amount of radioactivity in the esterified fatty acids will increase
d-	(___)	(___)	the amount of radioactivity in the glycerol fraction from saponified triacylglycerols will not change.

A – TRUE:

- **Adrenaline decreases the uptake of glucose and increases the use of fatty acids as a source of energy (instead of glucose).**

B – FALSE:

- **There will be less CO_2 produced from glucose and more from the fat depots; the CO_2 released will be less radioactive.**

C – FALSE:

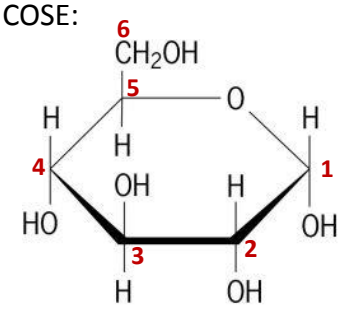
- **Adrenaline decreases lipogenesis from glucose as well as FA esterification.**

D – FALSE:

- **There will be less glucose used for TAG synthesis, so less radioactivity in the TAG-glycerol**

2. If instead of using (U-^{14}C) glucose you use glucose labelled in carbons 1 and 4, what would be the radioactivity ratio between the fatty acid and the glycerol fractions from the neutral lipids (≈triacylglycerols) saponification for the control sample A (no hormone)?

GLUCOSE:



- **During glycolysis, DHAP (carbons 1-3) and GAP (carbons 4-6) are produced from glucose. DHAP serves as a glycerol precursor. Therefore, if only 1 of the 3 carbons of DHAP is labelled, we would expect that the radioactivity in the glycerol fraction would be 1/3 less. Therefore:**

  radioactivity in the glycerol fraction = 1/3 of what you have obtained

- **Production of acetyl-CoA from pyruvate results in the loss of a carbon as CO_2. This carbon is derived from carbons 3/4 of the original glucose molecule. The remaining carbons (5/6 and 1/2) are incorporated into the fatty acid as two molecules of acetyl-CoA (used to make the intermediate malonyl-CoA). Since only one of the four carbons incorporated into the fatty acid chain are labelled (carbon 1 from the original glucose molecule), we would expect the radioactivity in the fatty acid fraction to be a quarter of what we obtain if all carbons are labelled. Therefore:**

  radioactivity in the fatty acid fraction = 1/4 of what you have obtained

- **The radioactivity ratio is calculated as follows:**
  - **FA (dpm)/ glyc (dpm) = (1/4)/(1/3) = <u>0.75 of what you have obtained</u>**

Lab 4: PEPTIDE FINGERPRINTING

1. The tryptic digestion of proteins in experiment 1 was performed over one hour incubation. However, when you incubate longer, you obtain the same peptide profile. How do you explain your observation?

After the one hour incubation trypsin has autodigested and there is no more protease activity left.

2. Briefly explain whether or not (and under which conditions) the technique of peptide fingerprinting can be used to test:

a) if a mutagenesis procedure has been successful
b) if enzymes from different tissues are isozymes (same function but different composition)
c) if a ligand binds to a protein
d) if a protein is affected by a denaturing agent

In each positive case, indicate whether partial or complete digestion should be used.

a) Yes, if the mutation modifies the tertiary structure of the protein. Complete digestion will not work unless the mutation removes or adds one or more proteolytic sites.

b) Yes, with partial digestion. Complete digestion will work if the AA sequences of the isozymes are different.

c) Yes if the bound ligand prevents the proteolytic cleavage at one or more sites. Complete digestion will not work.

d) Yes, if the protein denaturation changes the accessibility of one or more proteolytic sites. Complete digestion will not work.

3. Identify three experimental parameters of gel filtration HPLC which you can modify in order to increase the resolution of separation. Explain your answer.

1. Increasing the length of the column while keeping the column volume the same. This will increase the path length of the molecules across the matrix.

2. Decreasing the diameter of beads forming the matrix. This will increase the amount of matrix surface used in the column – it is equivalent to increasing the length of the column.

3. Decreasing the sample volume. This will decrease ω (peak width) and thus, increase the resolution.

Lab 5: LIGAND BINDING

1. For the calculation of **r** (degree of protein-ligand complexation), you measured ΔA at a certain wavelength, would you obtain the same **r** values if you measured ΔA at a different wavelength? Explain your answer.

Yes, because r is a function of the equilibrium constant and the relative concentrations of protein and ligand, but not of the wavelength used for the measurements.

2. Indicate whether the following statements about the interaction of BBG (Brilliant Blue G) with a protein are true or false. Explain your answers.

a) Binding of BBG causes a red shift of the absorption peak of BBG.

b) The anionic form of BBG binds non-covalently to the protein.

c) BBG binds to basic and hydrophobic residues.

d) Binding of BBG causes a hyperchromic effect (increase in absorption) at all wavelengths of the spectrum.

A – TRUE:

- **Compared with the free ligand, the bound ligand has its peak of absorption displaced towards the higher wavelength.**

B – TRUE:

- **Of the three ionic forms of BBG, it is the anionic form (blue) that is binding to the proteins. The binding causes a shift of the equilibrium between the three forms towards the anionic form.**

C – TRUE:

- **BBG has several aromatic rings that will interact with hydrophobic residues and, when in the anionic form, it will interact with positively charged residues.**

D – FALSE:

- **Upon binding, BBG spectrum increases in intensity at some wavelengths but decreases at other wavelengths.**

Lab 6: ALLOSTERIC BINDING

1. What would be the effect on $t_{intercept}$ and t_{final} of a) reducing the amount of yeast and b) increasing the amount of hemoglobin in your experiment?

a. **$t_{intercept}$ and t_{final} will both increase. Since there are fewer yeast cells, the available O_2 dissolved in solution is consumed less rapidly.**

b. **$t_{intercept}$ will remain constant because the oxygen consumption rate of the yeast cells doesn't change when we increase the amount of hemoglobin. However, there will be more O_2 available.**

t_{final} will increase due to the fact that there are more hemoglobin molecules that are releasing O_2 in the solution as the [O_2] decreases.

2. Normally arterial blood has a pO_2 = 100 torr, whereas venous blood has a pO_2 = 30 torr. In the blood, hemoglobin has a p_{50} = 26.5 torr. Assuming that the Hill equation with n = 3 is a good approximation, calculate the amount (as percentage of hemoglobin-bound oxygen) of O_2 unloaded from hemoglobin when the blood passes through the capillaries. Show your calculations. (Hill equation: $Y/(1-Y) = (pO_2/p_{50})^n$)

$Y/(1-Y) = (pO_2 / p_{50})^n$

Arteries: $Y/(1-Y) = (100/26.5)^3$, $Y = 0.98$

Veins: $Y/(1-Y) = (30/26.5)^3$, $Y = 0.59$

Unloaded O_2 : [(0.98-0.59)/0.98] x 100= 39.7%