18th INTERNATIONAL BIOLOGY OLYMPIAD
JULY 15 - 22, 2007

PRACTICAL EXAMINATION 3

Cell Biology/Biochemistry

TASK A.	**Thiocyanate analysis in cauliflower**	**27 marks**
TASK B.	**Determination of the amount of cauliflower needed to be consumed to cause toxicity**	**5 marks**
TASK C.	**Regulation of gene expression**	**18 marks**

Time allowed: 90 minutes

WRITE ALL ANSWERS IN THIS EXAM BOOKLET

WRITE YOUR 4-DIGIT STUDENT CODE IN THE BOX BELOW AND ON THE TOP OF EACH PAGE OF THIS BOOKLET

STUDENT CODE	

Introduction

The cabbage family contains a class of compounds known as glucosinolates. Some glucosinolates such as glucoraphanin have desired medicinal properties helping to prevent cancers while others such as glucosinalbin have toxic metabolites.

One of the products of the toxic glucosinolates is the thiocyanate ion (SCN^-). SCN^- interferes with iodine metabolism resulting in thyroid hormone deficiency. Eating plants of the crucifer family such as cauliflower will result in the production of a limited amount of thiocyanate ion from glucosinolates such as glucosinalbin.

The glucosinolate glucoraphanin is metabolized to sulforaphane. Sulforaphane is an inducer of phase 2 proteins. One consequence of phase 2 protein induction is an increased ability of cells to scavenge free radicals and other oxidants. A consequence of decreased oxidant levels is a lower probability of activation of pathways that lead to inflammation. One such pathway is through activation of a protein complex such as NFkappaB.

TASK A. To determine the amount of thiocyanate ion released from cauliflower using a spectrophotometric assay. (27 marks)

OBJECTIVE: To use a spectrophotometer to determine how much thiocyanate ion is released from cauliflower. This assay is based upon the principle that in an acid environment thiocyanate reacts with Fe^{3+} to form a stable Fe^{2+}-SCN red-coloured complex with a maximum absorption at 447 nm.

Materials

- Eppendorf pipettor: one 20-200 microlitre capacity set to 100 microlitres.
- Eppendorf pipette tips.
- Spectrophotometer cuvettes containing 900 microlitres of ferric nitrate reagent – as noted above, this reagent is in a strong acid.

  CAUTION: The ferric nitrate reagent solution you will be using is dissolved in 1.0 M nitric acid. Wear gloves and use goggles to protect your eyes before starting the experiment.

- Thiocyanate standards in tubes at the following concentrations: 0 micromoles/mL (this is your blank), 0.5 micromoles/mL, 1.0 micromoles/mL, 2.0 micromoles/mL and 4.0 micromoles/mL.
- One tube of filtered cauliflower homogenate. 1.0 g of cauliflower was homogenized and the homogenate was diluted to a total volume of 4.0 mL water. This is your unknown and you will be required to determine how many micromoles of thiocyanate are present in one millilitre of this homogenate.

- Marker pen to label the frosted side of each cuvette.
- Gloves and protective glasses
- On your bench is a spectrophotometer set to an absorbance of 447 nm.

NOTE: Before beginning this task, be sure that you have all the materials listed above. If you do not, notify a lab assistant by raising your hand.

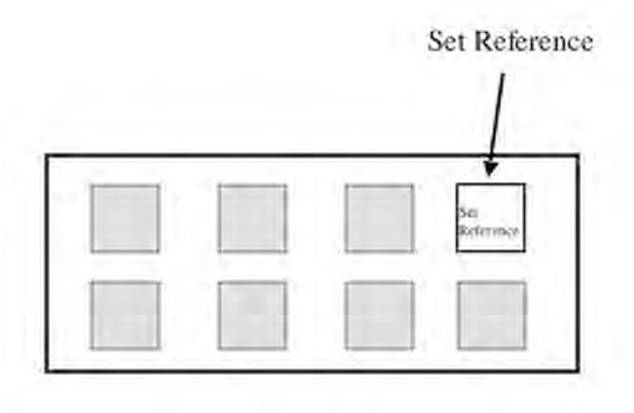
Procedure

1. Put on the gloves and the protective glasses.

2. To each of the cuvettes containing the ferric nitrate reagent add 100 microliters of each of the thiocyanate standards. The standards are: 0, 0.5, 1.0, 2.0 and 4.0 micromoles thiocyanate/mL. A coloured reaction should become visible except for the 0 micromole thiocyanate standard which serves as your blank. Be sure to label the cuvettes on the frosted surface.

3. To each of the remaining 3 cuvettes add 100 microlitres of the cauliflower homogenate.

4. Carefully carry the cuvettes to the spectrophotometer which has been set to absorb at 447 nm. Open the lid to the light path in the spectrophotometer and insert the 0 micromole thiocyanate/mL standard (i.e., blank) cuvette. **The arrow indicates the light path. Ensure that the walls of the cuvettes through which the light passes is transparent.**
   Close the lid and push the "set reference" button on the top right hand of the panel on the spectrophotometer – see the diagram below. **Do not touch any of the other buttons!**



5. Insert each of the standards and record the reading. Then insert each of the cuvettes containing the unknown and record the spectrophotometer reading. Leave the cuvettes at the spectrophotometer and the laboratory assistants will take care of them.

Spectrophotometer reading (absorbance) for each standard: (10 marks)

0.5 micromole/mL thiocyanate: _____

1.0 micromole/mL thiocyanate: _____

2.0 micromole/mL thiocyanate: _____

4.0 micromole/mL thiocyanate: _____

Spectrophotometer reading (absorbance) for the unknown: (4 marks)

1._____ 2._____ 3._____

6. Plot, on the graph paper (page 5), the absorbance measurements for your thiocyanate standards against the concentration (micromoles/mL) of the standards. (6 marks)

7. Calculate the average absorbance of your cauliflower homogenate. (2 marks)

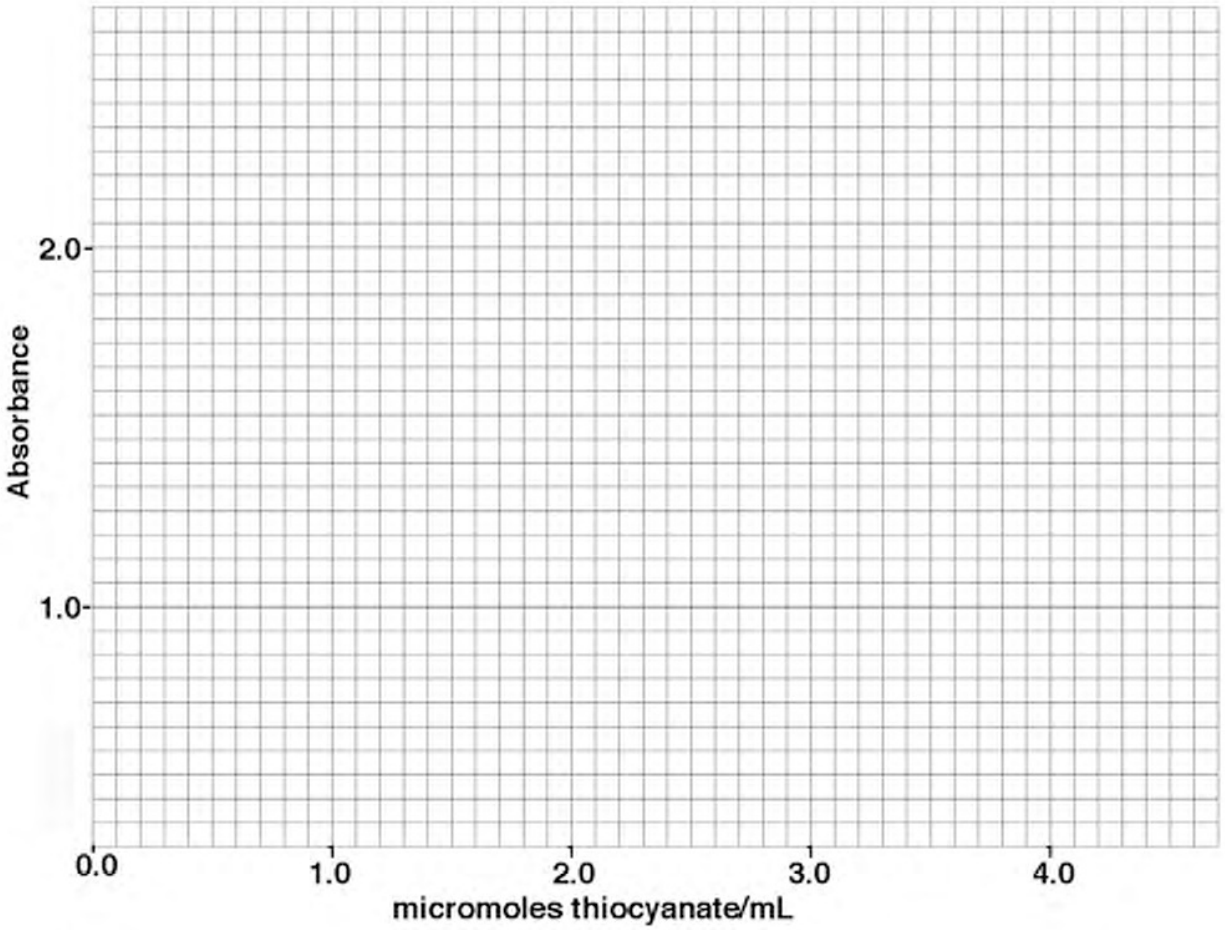
ANSWER: _____

8. What is the concentration of thiocyanate present in the cauliflower homogenate solution? (5 marks)

ANSWER: _____

9. What is the standard deviation of the absorbance of the unknown? (2 marks)

ANSWER: _____


Absorbance
2.0
1.0
0.0
1.0
2.0
3.0
4.0
micromoles thiocyanate/mL

TASK B. To determine the amount of cauliflower needed to be consumed for it to cause toxic effects because of the presence of thiocyanate (5 marks)

Introduction

The LD_{50} is a toxicology term that describes the dose (i.e., moles of toxin/kg animal) of a compound that will kill 50% of the animals tested. In the rat, the LD_{50} of sodium thiocyanate consumed is reported to be 9 millimoles/kg. Using the data of the experiment you have just performed, calculate how much cauliflower a rat that weighs 500 g would have to eat in a short time to reach the LD_{50} of thiocyanate.

Procedure

Circle the letter of the range that best fits your calculated value. Show your calculations on this page. Continue on the back of this page if necessary.

(a) 1 g to 5 g

(b) 50 g to 250 g

(c) 500 g to 1 kg

(d) 1.5 kg to 14 kg

(e) 15 kg to 25 kg

TASK C. To interpret the regulation of gene expression. (18 marks)

Introduction

The glucosinolate glucoraphanin is metabolized to sulforaphane. Sulforaphane is an inducer of phase 2 proteins. One consequence of phase 2 protein induction is an increased ability of cells to scavenge free radicals and other oxidants. A consequence of decreased oxidant levels is a lower probability of activation of pathways that lead to inflammation. One such pathway is through activation of a protein complex such as NFkappaB.

NFkappaB is a transcription factor complex comprised of two proteins (p50 and p65) bound to a third protein known as IkappaB that is normally present in the cytoplasm. Activation of NFkappaB involves the degradation of IkappaB resulting in the NFkappaB p50/p65 heterodimer translocating to the nucleus where it binds to specific promoter elements increasing the transcription of pro-inflammatory genes such as inducible nitric oxide synthase (iNOS). One indicator of activation of NFkappaB is that the ratio of the p65 to IkappaB protein increases.

One of the consequences of increased iNOS activity is excessive production of the nitric oxide free radical ($NO^{\cdot}$). Nitric oxide reacts with the superoxide anion ($O_2^{\cdot-}$) to form peroxynitrous acid. Peroxynitrous acid is a very strong oxidant.

Increased oxidant levels often results in activation of NFkappaB while lowering oxidant levels often results in decreased activation of NFkappaB and, hence, lowered levels of expression of pro-inflammatory genes.

Procedure

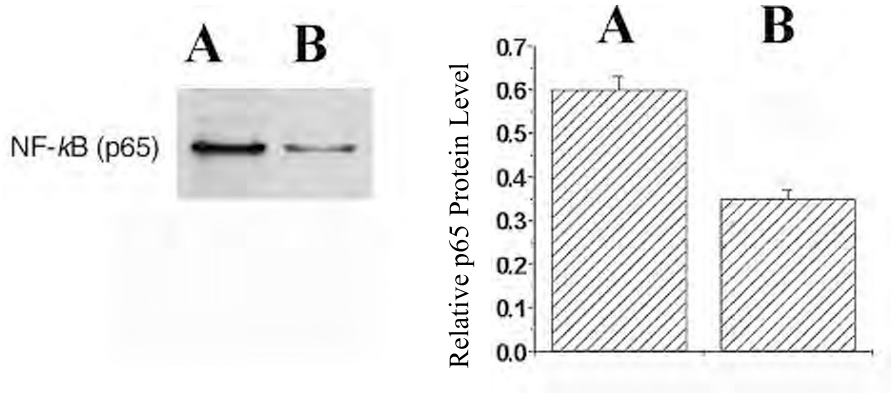
1. Examine the figures provided in each of the following sections.

2. Using the data presented, identify which data set is derived from animals fed a diet high in glucoraphanin and provide the basis for your answer.

SECTION A. (5 marks)
Below is a figure that gives data on NFkappaB activation in spontaneously hypertensive stroke-prone (SHRsp) male rats that were fed one of two diets: a control diet or an experimental diet containing glucoraphanin. In the experimental diet, the animals consumed 10 micromoles glucoraphanin/kg body weight.

After several months on these diets, the animals were euthanized, nuclei from the kidney cells were isolated and prepared for SDS polyacrylamide electrophoresis. Following separation of the proteins on the gel, the proteins were transferred to nitrocellulose membrane and probed with an antibody that recognized the NFkappaB p65 protein.

A representative Western blot is shown below (on the left) and next to it is a graph that depicts the quantification of blots from 5 different animals per diet group.



Answer the following questions:

1. Which group of animals (A or B) were on the glucoraphanin-containing diet? (1 mark)

ANSWER: _____

2. Which of the following statements gives the best explanation for your answer? Circle the letter of that statement. (4 marks)

(a) Less oxidative stress results in less NFkappaB activation and hence less p65 in the nuclei.

(b) Less oxidative stress results in less NFkappaB activation and hence more p65 in the nuclei.

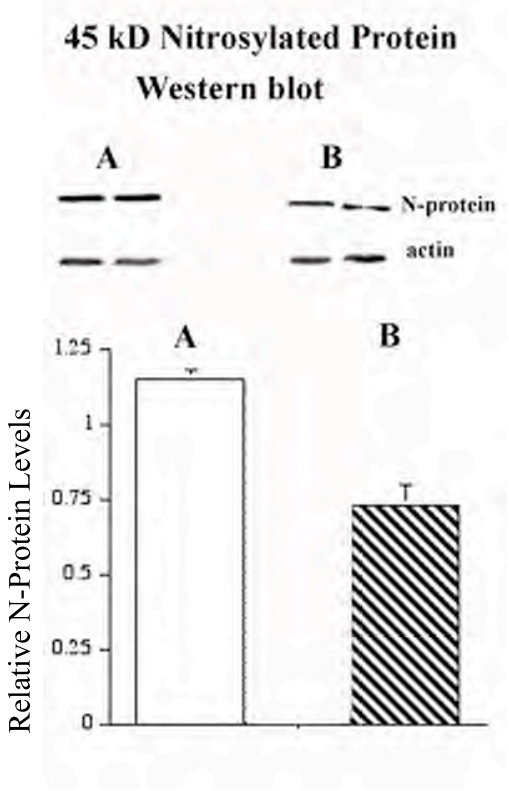
(c) More oxidative stress results in less NFkappaB activation and hence less p65 in the nuclei.

(d) More oxidative stress results in less NFkappaB activation and hence more p65 in the nuclei.

(e) More oxidative stress results in more NFkappaB activation and hence less p65 in the nuclei.

SECTION B. (8 marks)

Below is a figure that gives Western blot data on a 45 kD nitrosylated protein (N-protein) in the kidneys of two male SHRsp rats that were put on one of two different diets: a diet containing glucoraphanin and control diets.



The top part of the figure is a representative Western blot while the bottom part of the figure is the quantification of Western blots from 5 different animals per diet group.

Answer the following questions:

1. Which group (A or B) represents the animals fed a diet containing glucoraphanin? (1 mark)

ANSWER: _____

2. Circle the letter of the statement below that best explains your answer. (4 marks)

(a) More oxidative stress results in more NFkappaB activation that results in more iNOS expression and more peroxynitrous acid formation and thus more nitrosylation of proteins.

(b) More oxidative stress results in more NFkappaB activation that results in more iNOS expression and more peroxynitrous acid formation but less nitrosylation of proteins.

(c) More oxidative stress results in more NFkappaB activation that results in more iNOS expression but less peroxynitrous acid formation and thus less nitrosylation of proteins.

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(e) Less oxidative stress results in less NFkappaB activation that results in less iNOS expression and less peroxynitrous acid formation and thus less nitrosylation of proteins.

3) The figure above shows the amount of an additional protein, actin. Why is the level of this protein measured? (3 marks)

a) To quantify the expression of N-protein relative to a protein that is assumed to be equally expressed in kidney cells independent of the treatment.

b) To show that the expression level of N-protein is independent of the effect of the treatment on the filtration rate of the kidney.

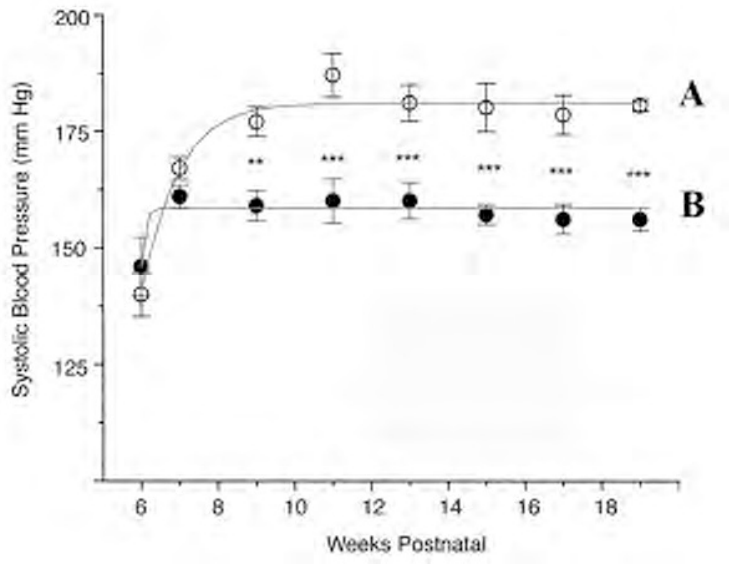
c) To ensure that N-protein and actin are not bound to each other in the cells of rats that received the treatment.

d) The level of a protein whose expression is assumed to be independent of the treatment is measured to ensure that the binding of the antibody used to detect N-protein is unaffected by the treatment.

SECTION C. (5 marks)

The nitric oxide radical ($NO^{\cdot}$), that is released by endothelial cells of blood vessels, diffuses to smooth muscle cells where it causes smooth muscle cells to relax and thus decreases blood pressure. The amount of $NO^{\cdot}$ that can diffuse to the smooth muscle depends upon the overall level of superoxide radicals. If there are excessive superoxide radicals present, then $NO^{\cdot}$ reacts with superoxide forming peroxynitrous acid.

Below is a graph depicting the systolic blood pressures of female SHRsp rats placed on one of two diets, a control diet and a diet containing glucoraphanin. Animals were placed on these diets at the age of 5 weeks post-natal and blood pressures were monitored from 6 to 19 weeks post-natal.



Answer the following questions:

1. Which group of animals, A or B, were fed the glucoraphanin-containing diet? (1mark)

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- THE END –

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One of the products of the toxic glucosinolates is the thiocyanate ion (SCN^-). SCN^- interferes with iodine metabolism resulting in thyroid hormone deficiency. Eating plants of the crucifer family such as cauliflower will result in the production of a limited amount of thiocyanate ion from glucosinolates such as glucosinalbin.

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- On your bench is a spectrophotometer set to an absorbance of 447 nm.

NOTE: Before beginning this task, be sure that you have all the materials listed above. If you do not, notify a lab assistant by raising your hand.

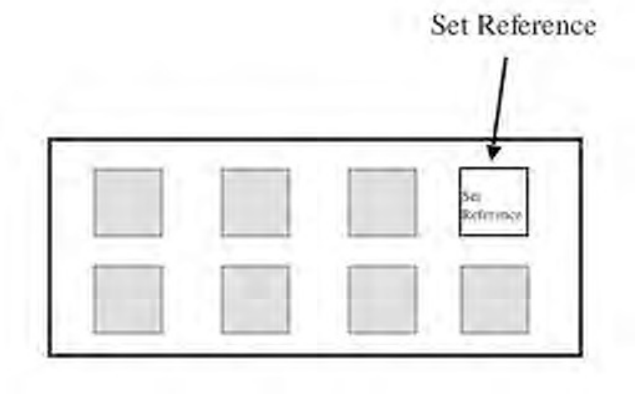
Procedure

1. Put on the gloves and the protective glasses.

2. To each of the cuvettes containing the ferric nitrate reagent add 100 microliters of each of the thiocyanate standards. The standards are: 0, 0.5, 1.0, 2.0 and 4.0 micromoles thiocyanate/mL. A coloured reaction should become visible except for the 0 micromole thiocyanate standard which serves as your blank. Be sure to label the cuvettes on the frosted surface.

3. To each of the remaining 3 cuvettes add 100 microlitres of the cauliflower homogenate.

4. Carefully carry the cuvettes to the spectrophotometer which has been set to absorb at 447 nm. Open the lid to the light path in the spectrophotometer and insert the 0 micromole thiocyanate/mL standard (i.e., blank) cuvette. Note that the clear walls of the cuvette should be in line with the arrow indicators in the spectrophotometer chanber.
   Close the lid and push the "set reference" button on the top right hand of the panel on the spectrophotometer – see the diagram below.
   Do not touch any of the other buttons!



5. Insert each of the standards and record the reading. Then insert each of the cuvettes containing the unknown and record the spectrophotometer reading. Leave the cuvettes at the spectrophotometer and the laboratory assistants will take care of them.

Spectrophotometer reading (absorbance) for each standard: (10 marks)

0.5 micromole/mL thiocyanate: _____

1.0 micromole/mL thiocyanate: _____

2.0 micromole/mL thiocyanate: _____

4.0 micromole/mL thiocyanate: _____

Spectrophotometer reading (absorbance) for the unknown: (3 marks)

1._____ 2._____ 3._____

6. Plot, on the graph paper (page 5), the absorbance measurements for your thiocyanate standards against the concentration (micromoles/mL) of the standards. (6 marks)

7. Take the average absorbance of your cauliflower homogenate and determine the thiocyanate ion concentration using the previously plotted graph. (5 marks)

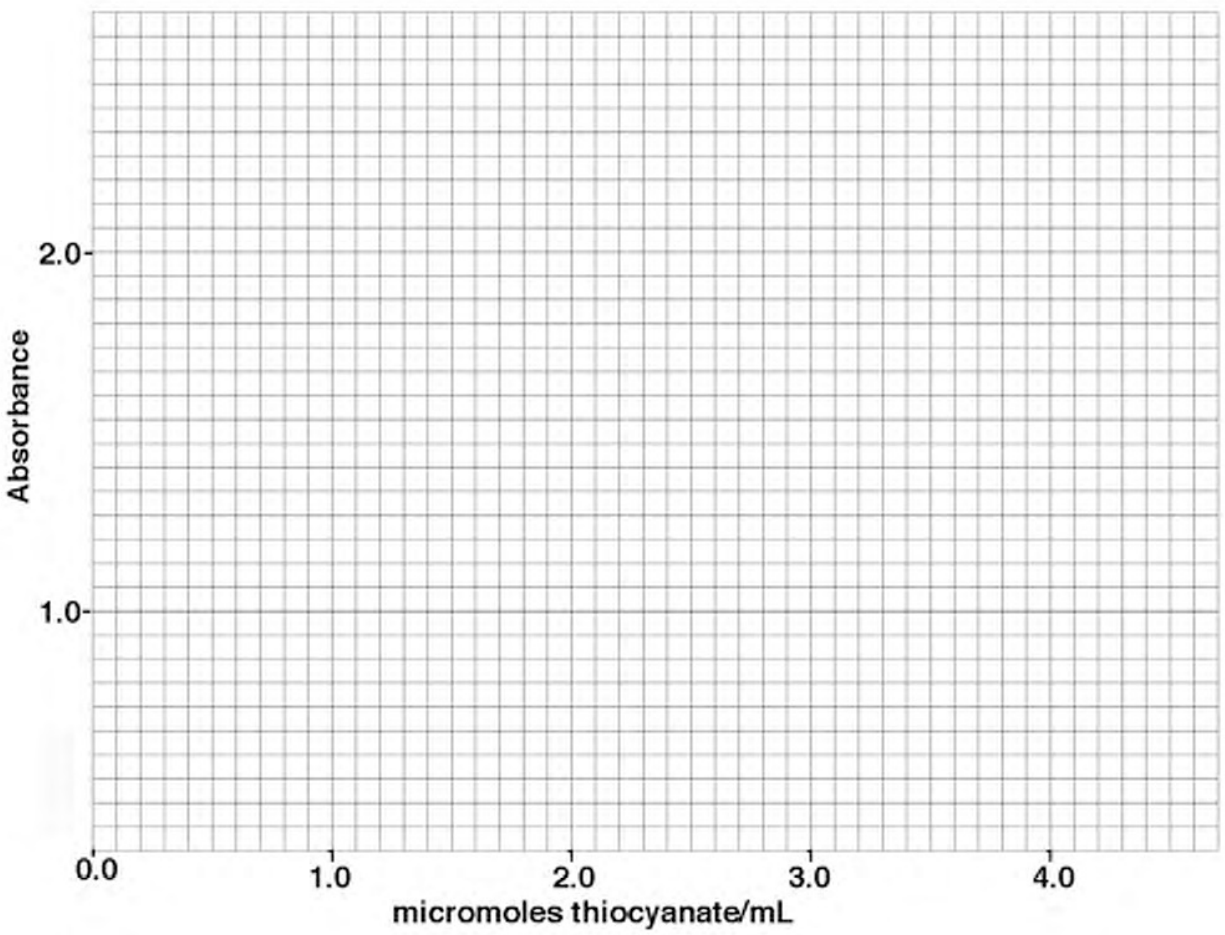
ANSWER: _____

8. What is the concentration of the thiocyanate ion present in your cauliflower homogenate? Be sure to state the units. (3 marks)

ANSWER: _____

9. Calculate the standard deviation of the spectrophotometer reading of the unknown? (2 marks)

ANSWER: _____


Absorbance
2.0
1.0
0.0
1.0
2.0
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micromoles thiocyanate/mL

TASK B. To determine the amount of cauliflower needed to be consumed for it to cause toxic effects because of the presence of thiocyanate (5 marks)

Introduction

The LD_{50} is a toxicology term that describes the dose (i.e., moles of toxin/kg animal) of a compound that will kill 50% of the animals tested. In the rat, the LD_{50} of sodium thiocyanate consumed is reported to be 9 millimoles/kg. Using the data of the experiment you have just performed, calculate how much cauliflower a rat that weighs 500 g would have to eat in a short time to reach the LD_{50} of thiocyanate.

Procedure

Circle the letter of the range that best fits your calculated value. Show your calculations on this page. Continue on the back of this page if necessary.

(a) 1 g to 5 g

(b) 50 g to 250 g

(c) 500 g to 1 kg

(d) 1.5 kg to 14 kg

(e) 15 kg to 25 kg

TASK C. To interpret the regulation of gene expression. (18 marks)

Introduction

The glucosinolate glucoraphanin is metabolized to sulforaphane. Sulforaphane is an inducer of phase 2 proteins. One consequence of phase 2 protein induction is an increased ability of cells to scavenge free radicals and other oxidants. A consequence of decreased oxidant levels is a lower probability of activation of pathways that lead to inflammation. One such pathway is through activation of a protein complex such as NFkappaB.

NFkappaB is a transcription factor complex comprised of two proteins (p50 and p65) bound to a third protein known as IkappaB that is normally present in the cytoplasm. Activation of NFkappaB involves the degradation of IkappaB resulting in the NFkappaB p50/p65 heterodimer translocating to the nucleus where it binds to specific promoter elements increasing the transcription of pro-inflammatory genes such as inducible nitric oxide synthase (iNOS). One indicator of activation of NFkappaB is that the ratio of the p65 to IkappaB protein increases.

One of the consequences of increased iNOS activity is excessive production of the nitric oxide free radical ($NO^{\cdot}$). Nitric oxide reacts with the superoxide anion ($O_2^{\cdot-}$) to form peroxynitrous acid. Peroxynitrous acid is a very strong oxidant.

Increased oxidant levels often results in activation of NFkappaB while lowering oxidant levels often results in decreased activation of NFkappaB and, hence, lowered levels of expression of pro-inflammatory genes. The nitric oxide that is released by endothelial cells diffuses to smooth muscle cells where it causes smooth muscle cells to relax. Hence, nitric oxide is a major regulator of blood pressure.

Procedure

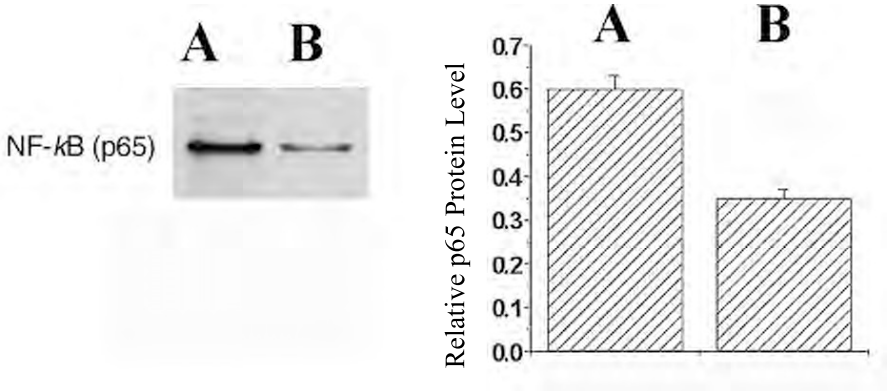
1. Examine the figures provided in each of the following sections.

2. Using the data presented, identify which data set is derived from animals fed a diet high in glucoraphanin and provide the basis for your answer.

SECTION A. (5 marks)
Below is a figure that gives data on NFkappaB activation in spontaneously hypertensive stroke-prone (SHRsp) male rats that were fed one of two diets: a control diet or an experimental diet containing glucoraphanin. In the experimental diet, the animals consumed 10 micromoles glucoraphanin/kg body weight.

After several months on these diets, the animals were euthanized, nuclei from the kidney cells were isolated and prepared for SDS polyacrylamide electrophoresis. Following separation of the proteins on the gel, the proteins were transferred to nitrocellulose membrane and probed with an antibody that recognized the NFkappaB p65 protein.

A representative Western blot is shown below (on the left) and next to it is a graph that depicts the quantification of blots from 5 different animals per diet group.



Answer the following questions:

1. Which group of animals were on the glucoraphanin-containing diet? The group represented by A or B? (1 mark)

ANSWER: _____**B**_____

2. Which of the following statements gives the best explanation for your answer? Circle the letter of that statement. (4 marks)

(a) Less oxidative stress results in less NFkappaB activation and hence less p65 in the nuclei.

(b) Less oxidative stress results in less NFkappaB activation and hence more p65 in the nuclei.

(c) More oxidative stress results in less NFkappaB activation and hence less p65 in the nuclei.

(d) More oxidative stress results in less NFkappaB activation and hence more p65 in the nuclei.

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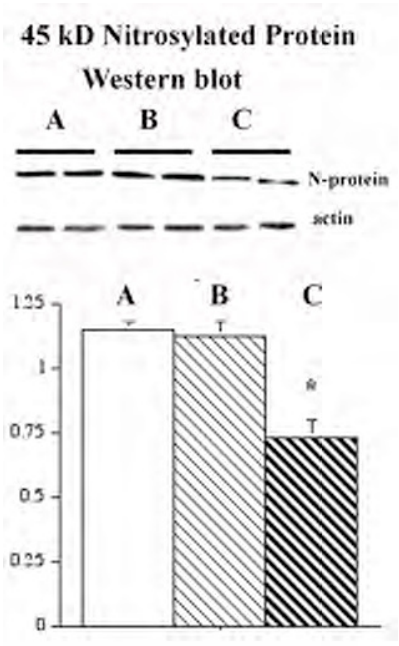
SECTION B. (5 marks)

Below is a figure that gives Western blot data on a 45 kD nitrosylated protein (N-protein) in the kidneys of two male SHRsp rats that were put on one of two different diets: a diet containing glucoraphanin and control diets.



The top part of the figure is a representative Western blot while the bottom part of the figure is the quantification of Western blots from 5 different animals per diet group.

Answer the following questions:

1. Which group represents the animals fed a diet containing glucoraphanin? (1 mark)

ANSWER: _____C_____

2. Circle the letter of the statement below that best explains your answer. (4 marks)

(a) More oxidative stress results in more NFkappaB activation that results in more iNOS expression and more peroxynitrous acid formation and thus more nitrosylation of proteins.

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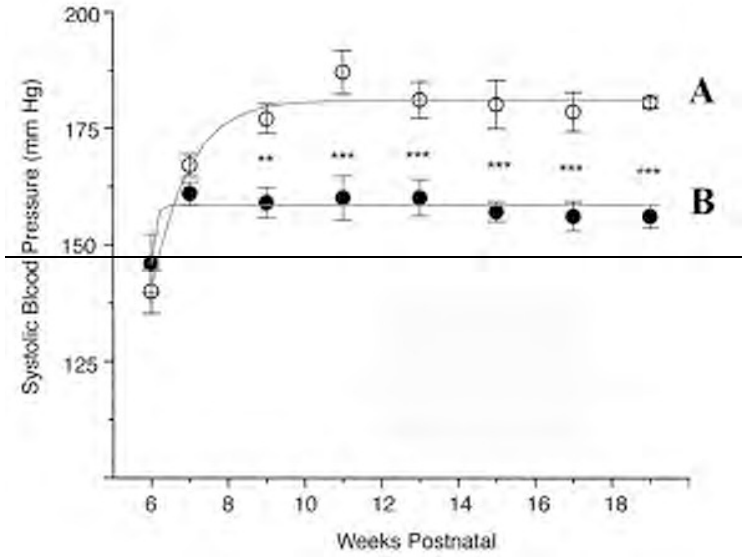
(d) More oxidative stress results in less NFkappaB activation but results in less iNOS expression and less peroxynitrous acid formation and thus less nitrosylation of proteins.

(e) Less oxidative stress results in less NFkappaB activation that results in less iNOS expression and less peroxynitrous acid formation and thus less nitrosylation of proteins.

~~**SECTION C**. (5 marks)~~

~~The nitric oxide radical ($NO^{\cdot}$), that is released by endothelial cells of blood vessels, diffuses to smooth muscle cells where it causes smooth muscle cells to relax and thus decreases blood pressure. The amount of $NO^{\cdot}$ that can diffuse to the smooth muscle depends upon the overall level of superoxide radicals. If there are excessive superoxide radicals present, then $NO^{\cdot}$ reacts with superoxide forming peroxynitrous acid.~~

~~Below is a graph depicting the systolic blood pressures of female SHRsp rats placed on one of two diets, a control diet and a diet containing glucoraphanin. Animals were placed on these diets at the age of 5 weeks post-natal and blood pressures were monitored from 6 to 19 weeks post-natal.~~



~~Answer the following questions:~~

~~1. Which group of animals, A or B, were fed the glucoraphanin-containing diet? (1mark)~~

~~_____~~

~~ANSWER: _____~~

~~2. Circle the letter of the statement below that best explains your answer. (4 marks)~~

~~(a) Less oxidative stress results in lower scavenging of nitric oxide. Decreased amounts of nitric oxide available to vascular smooth muscle results in more vasorelaxation and thus lower blood pressure.~~

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- THE END –

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