

Project One: Identification of unknown mutants in the arginine biosynthetic pathway of *Neurospora crassa* through growth tests and measurements of levels of intermediates

Overview

In this lab, unknowns will be provided that are either have a complete set of functional arginine biosynthetic enzymes (wild type) or are defective in one of these enzymes. The goal in the project is to identify these unknowns as being either wild type or one of the arg mutants which include *arg1*, *arg6*, *arg10*, *arg12* and *arg14* (for fungus, the mutant version of a gene is listed in italics while the normal version is not). This will be done using two methods: growth tests and measurement of level of intermediates. In the growth tests, the unknowns are inoculated onto minimal medium which contains either none or one of the arginine biosynthetic pathway intermediates. It is expected that mutants will grow in those intermediates that come after the blocked enzyme reaction but not those that come before the block. For the level of intermediates, the unknowns will be grown, amino acids will be extracted and separated by cation exchange chromatography and then assayed in colorimetric tests. It is expected that the mutants will show a decreased level of those intermediates that come after the blocked step and an increased level for those that come before the blocked step compared to wild type. In order to separate the amino acids by cation exchange chromatography, it is first necessary to identify where each amino acid elutes from the column. This is done by passing standard mixtures of all three amino acids over the column, collecting all fractions and testing for the three amino acids in separate colorimetric tests. The results from both growth tests and levels of intermediates can be used to determine the identity of the unknowns.

Introduction to growth tests and levels of intermediates

In this experiment, an amino acid biosynthetic pathway in the fungus *Neurospora crassa* will be characterized. *N. crassa* represents a simple type of eukaryotic cell. Its use has long been favored for use in biochemical and genetic experiments for several reasons. The medium used for growth is simple, fairly inexpensive, and easy to prepare. As a simple eukaryote, some of the information learned in *N. crassa* can be extrapolated to more complex eukaryotes, such as mammalian cells. There are a large number of existing mutants in *N. crassa* that have been previously characterized. A mutation is caused by a change in the nucleotide sequence of DNA sequence for the gene that encodes the protein. This results in a change in the amino acid sequence of the protein which prevents the protein from assuming the correct three dimensional shape. A protein which lacks the proper conformation does not function properly; if the protein is an enzyme, there is a complete or partial loss of activity depending on the severity of the mutation. Beadle and Tatum took advantage of the many *N. crassa* mutants to prove the one gene, one enzyme hypothesis. While we take this fact for granted now, Beadle and Tatum originally proved this to be the case by looking at mutants in the pathway we are characterizing in this project.

A hypothetical biosynthetic pathway is shown below.



The letters represent intermediates and each arrow represents an enzyme that catalyzes the biochemical reaction. These enzymes are proteins that are ultimately encoded by genes which can be mutated. If there is a mutation in the gene for the third enzyme which converts C to D,

what happens to the level of intermediates compared to the wild type? This mutant is unable to convert D to E; consequently the levels of E as well as F are decreased compared to the wild type. The level of D is increased and as this occurs, the reaction catalyzed by enzyme 3 can be run in the reverse direction and the level of C can build up compared to wild type. The levels of A and B in the mutant might also be increased compared to wild type. Remember enzymes influence only the rate of a reaction and not the extent of a reaction—that is the ratio of product to substrate at equilibrium. As the product levels increase, this causes the reaction to proceed in the direction of reactants to restore the equilibrium ratio.

Similarly, a mutant in the second enzyme would accumulate B while the levels of C, D, E and F compared to the wild type would decrease. The levels always have to be compared to the wild type. The relative levels of intermediates in the wild type depend on the kinetics and thermodynamics of the reactions. The results for measuring the accumulation of intermediates are shown below where a + means the level is increased compared to wild type while a – means the level is decreased compared to wild type.

Mutant	Level of Intermediate compared to wild type					
	A	B	C	D	E	F
1	+	-	-	-	-	-
2	+	+	-	-	-	-
3	+	+	+	-	-	-
4	+	+	+	+	-	-
5	+	+	+	+	+	-

Thus, this experiment reveals a great deal about the enzymes and intermediates involved in a biochemical pathway. In order to be able to carry out this experiment, it is necessary to grow the various mutants, extract the intermediates and be able to measure them. One intermediate cannot interfere with the measurement of another.

Another way of characterizing the mutants in a pathway is to do growth tests. In the pathway shown, all of the mutants are blocked at various steps. In a biosynthetic pathway, this leads to an auxotrophic condition—that is, the mutants are unable to grow in minimal medium and require supplementation. Since the mutant strains are unable to synthesize the end product, all of them grow if it is provided in the medium. Depending on the position of the mutation in the pathway, the mutants will also grow if other intermediates are provided. In the pathway shown above, all of the mutant strains will grow if F is supplied. Since the mutant in the fifth enzyme is blocked in the conversion of E to F, it will grow only in the presence of F. Providing the mutant with E or any intermediate before E cannot support growth. The mutant that is blocked in the fourth enzyme cannot carry out the conversion of D to E and will grow if either F or E is supplied. A series of unknown mutants can be ordered based on the growth tests. The results for growth tests for mutants in the pathway shown above is in the table shown below where a + means that growth occurred and – means there was no growth.

Mutant	Growth in intermediate					
	A	B	C	D	E	F
1	-	+	+	+	+	+
2	-	-	+	+	+	+
3	-	-	-	+	+	+
4	-	-	-	-	+	+
5	-	-	-	-	-	+

Thus, the pattern that emerges is that the mutants are unable to grow in minimal medium alone but if provided with an intermediate that occurs after the blocked step, the mutant can grow. It is possible to deduce the order of a pathway as well as the position of the mutated enzymes by growing in the presence of the various intermediates. In order to do this method, it is necessary that the intermediates are available. Some pathways contain highly unstable intermediates that break down quickly and these are generally not readily available. The information gained from the growth test should complement that of the level of intermediates and both reveal the position of enzymes and intermediates in a biochemical pathway.

Introduction to the arginine biosynthetic pathway in *Neurospora crassa*

In animals, arginine is generally considered to be an essential amino acid. While there is a pathway with enzymes to synthesize arginine, it generally does not produce enough arginine to supply the needs and thus, it must come from the diet. Like many lower eukaryotes, there are no essential amino acids in *Neurospora crassa*—it is able to synthesize all that is needed. The arginine biosynthetic pathway has long been of interest for several reasons. It was the pathway used by Beadle and Tatum to prove that one gene encodes one enzyme. In addition, some of the enzymes in the pathway are seen in the urea cycle, a pathway used in terrestrial animals as way of getting rid of the nitrogen that comes from amino acids in proteins.

The complete arginine biosynthetic pathway in *Neurospora* is shown below:

Glutamate $\xrightarrow{\quad}$ N-acetyl glutamate $\xrightarrow{\quad}$ N-acetyl- γ -glutamyl phosphate $\xrightarrow{\quad}$ N-acetyl- γ -glutamate semialdehyde $\xrightarrow{\quad}$ N-acetyl ornithine $\xrightarrow{\quad}$ ornithine $\xrightarrow{\quad}$ citrulline $\xrightarrow{\quad}$ argininosuccinate $\xrightarrow{\quad}$ arginine

A number of these intermediates are unstable and for our purposes, the pathway can be simplified to

Glutamate $\xrightarrow{\quad}$ $\xrightarrow{\quad}$ $\xrightarrow{\quad}$ ornithine $\xrightarrow{\quad}$ citrulline $\xrightarrow{\quad}$ $\xrightarrow{\quad}$ arginine

where each arrow represents a unique enzyme in the pathway that is encoded by a gene. Arg6 is the gene that encodes acetyl glutamate kinase which catalyzes the formation of N-acetyl glutamyl phosphate and arg14 encodes acetyl glutamate synthase or the enzyme that makes N-acetyl glutamate. Thus, both arg 6 and arg 14 are involved in the conversion of glutamate to ornithine. The defective forms of these enzymes which are known respectively as *arg6* and *arg14*. Arg 12 encodes ornithine transcarbamylase which is the enzyme that converts ornithine

to citrulline. Only one enzyme is needed for this conversion and a mutant version is available. *Arg1* encodes arginosuccinate synthase which converts citrulline to arginosuccinate and *arg10* encodes arginosuccinate lyase which forms arginine. Thus, both of these enzymes are involved in the conversion of citrulline to arginine. The table shown below summarizes the mutants used in this project.

Strain used in the project	Blocked in the formation of what intermediate
74A or wild type	none
<i>Arg6</i>	Ornithine
<i>Arg14</i>	ornithine
<i>Arg12</i>	citrulline
<i>Arg1</i>	arginine
<i>Arg10</i>	arginine

You will be given the strains shown above as unknowns; you will receive strains A through F. Your goal in the project is to identify these strains as one of the six possibilities in the table shown above. This will be done using two methods: growth tests on the intermediates and measurement of levels of intermediates compared to wild type.

In order to analyze the results from levels of intermediates, all of the intermediates are compared to wild type and thus, it is necessary to know which strain is the wild type. The only way to obtain this information is from the growth tests. Since the wild type is the only strain with a completely functional set of enzymes, it is the only strain that can grow with none of the intermediates added. Thus, in order to interpret results from the level of intermediates, the growth test must be done.

In order to carry out the measurements of the intermediates, it is necessary to be able to separate a mixture of the amino acids using cation exchange chromatography. The columns are provided as well as the method for elution of amino acids. It is unknown which elution fraction contains which amino acid. Thus, it is necessary to first pass a mixture of known amounts of the three amino acids over the column, collect all fractions and assay them for each of the three amino acids using separate colorimetric tests. Then, it is possible to identify where each amino acid elutes and it is only necessary to collect the appropriate fractions.

Thus, to carry out this project there is essentially three experiments: growth tests, calibration of the columns and measurement of levels of intermediates. These experiments will be done concurrently and the instructions to do so are described later. Some background information on the growth tests and levels of intermediates is provided.

Growth tests for the arginine biosynthetic pathway

In order to do the growth tests, you will have to prepare sterilized solid medium that contains the appropriate intermediates, inoculate with the six unknown strains and after an appropriate period of time, observe whether there is growth or not. The available intermediates in the pathway are arginine, citrulline, glutamate and ornithine. Growth will also be measured in unsupplemented medium. Minimal medium for *N. crassa* is referred to as Vogel's minimal (VM) and contains 1 X Vogel's salts and 1.5 % sucrose as a carbon source. In order to make this solid, 2% agar is added. The Vogel's salts contains those mostly inorganic compounds that *Neurospora* needs to grow including a nitrogen and phosphorous source as well as minerals needed. It is prepared as a 50 X solution which means that it is 50 times as concentrated as needed and must be diluted before use. It also contains the one vitamin that *Neurospora* cannot synthesize—biotin. The solid VM is sterilized using the autoclave which results in greatly increased temperatures and pressures and kills even the highly resistant endospores of bacteria. After medium is prepared, you will inoculate the sterile medium with each of the strains for each type of medium and after appropriate time, check for growth. It is imperative that you use sterile techniques and keep good records.

Level of intermediates in *Neurospora*

The other major component in this project is to measure the level of the three amino acids in the mutants and compare to wild type. In order to do this, it is necessary to collect the amino acids from *N. crassa*, separate them to prevent interference, and measure the level of each using a separate colorimetric test. The amino acids are separated using a Dowex cation exchange column. This column contains polystyrene which acts as a large support molecule to which is bound an SO_3^{-2} group and this will repel negatively charged species while retaining those that are positively charged. Two of the three of the amino acids that will be measured—arginine and ornithine are positively charged at pH 7.0. In order to elute the amino acids, it is necessary to change the pH and thus the charge on the amino acids.

It is first necessary to calibrate the column and this is done by passing known amounts of amino acids over the column and following the elution schedule. All fractions are tested for the three amino acids by the different colorimetric tests: Sakaguchi test is used for arginine, Koritz-Cohen for citrulline and Chinard test for ornithine. Where each amino acid elutes from the column as well as the % recovery will be determined. The location of elution will be used to collect the appropriate fractions when the levels of intermediates are measured and these values will be corrected using % recovery.

In order to measure the level of intermediates, it is necessary to grow each strain. Since five of the six strains are auxotrophs, they will not grow in minimal medium and are supplemented with a limited amount of arginine. *N. crassa* is collected by filtration and broken open by boiling water bath extraction. This method will certainly denature proteins and cause them to precipitate; since the goal of the experiment is to collect amino acids and not functional proteins, this extreme method can be used. After the boiling water bath, there is a centrifugation step and the supernatant which contains the amino acids and other metabolites is collected and a portion is passed over the cation exchange column. Only those fractions which contain the three amino acids of interest are collected and measured for the level of each amino acid using the appropriate colorimetric test. The pellet contains the proteins and other macromolecules—it is used to measure the total level of protein. The level of intermediates is reported in nmole/mg protein and all of the values are compared to wild type.

Instructions

1. In this project, you will collect data from three types of experiments and this is summarized in the table below.

Type of experiment	Results produced	What is done with results?
Growth tests	Intermediates that did and did not support growth of unknowns	Determine identity of unknowns
Calibration of columns	Identification of which fraction contains the amino acid and % recovery for each amino acid	Location is used to collect appropriate fraction and % recovery to correct cellular pool values
Level of intermediates	Nmole/mg protein for arginine, ornithine and citrulline	Determine identity of unknowns

2. The order in which these tests will be done is important and must follow a certain sequence. Some of these experiments can be done simultaneously.
3. Each of these experiments also requires a certain sub set of tasks to be done and these are summarized in the table shown.

Type of experiment	Tasks that are required
Growth tests	<ol style="list-style-type: none"> 1. preparation of solid medium(slants) 2. inoculation of solid medium(slants)
Calibration of columns	Calibration of columns
Level of intermediates	<ol style="list-style-type: none"> 1. preparation and inoculation of liquid medium 2. measuring pools of arginine, ornithine and citrulline

3. It is recommended that the growth tests and calibration of columns can begin immediately and be done simultaneously. Two trials should be done for level of intermediates but this experiment requires usable results from the column calibration experiment.
4. The instructions for the three types of experiments are described.

Calibration of cation exchange columns.

In order to do this, you will need

1. Dowex cation exchange columns that have been prepared so that the elution schedule takes 2.0 ml for each wash. These columns contain Dowex 50 W, 200 –400 mesh as the cation exchange material.
2. 10 mM solutions of arginine, citrulline and ornithine
3. 0.116 M Na₃ citrate pH 5.3 and 0.2 M NaOH

1. Prepare solutions for elution: the citrate buffer and NaOH solution

Prepare 1.0 L of 0.116 M citrate, pH 5.3 and 1.0 L of 0.2 M NaOH.

For the citrate buffer, use citrate trisodium salt (Na₃citrate 2 H₂O, MW=294.10g/mole) and adjust the pH to 5.3 with a solution of 4.0 M HCl.

You will have to use a pH meter and it has to be calibrated before use. Make sure that you consult the teaching assistant or the instructor.

2. Prepare a mixture of arginine, ornithine and citrulline from 10mM standard solutions. The standard solutions will be prepared for you. From the standard solutions, remove a volume equal to 5 μmole and transfer to test tube. Do this for arginine, ornithine and citrulline. Bring the total volume of the mixture up to 2.0 mL with the sodium citrate buffer. Prepare six standard mixtures.

3. Apply the six mixtures to the column and begin the elution schedule. The columns have been prepared for you. Remove both the white cap and the yellow plug at the bottom. Allow the remaining water on the column to drain. When there is no liquid visible on top of the column, the mixtures can be added

4. Collect the amino acids by using the elution schedule

1. Apply samples to column.
2. 2.0 mL of sodium citrate
3. 2.0 ml of sodium citrate
4. 2.0 ml of sodium citrate
5. 2.0 ml of sodium citrate
6. 2.0 mL of 0.2 M NaOH
7. 2.0 ml of 0.2 M NaOH

After the mixtures have entered the column and there is no visible liquid left, the first 2.0 ml wash of the citrate buffer can be added. A test tube should be set up to collect this as the first fraction. When there is no more visible liquid left, the test tube should be removed and replaced with another and the second citrate buffer can be added. In this way, six fractions should be collected from each column with a volume of 2.0 ml. It is imperative that the fractions are labeled clearly. The labeling should include a way of naming each column as well as the number of the fraction.

5. When you have collected the last fraction, add 5 ml of distilled water to the column and allow it to elute. You don't have to collect this fraction. This is necessary to clean the column. When the water has drained, put the yellow cap back on the bottom and the white plastic cap on the top. Add 2.0 ml of distilled water to the column. The columns can be stored in this form indefinitely.

8. Test each fraction for the presence of all three amino acids using individual colorimetric tests. Each amino acid is measured using a separate colorimetric test that is described at the end of this project: Sakaguchi test for arginine, Chinard test for Ornithine and Koritz-Cohen test for citrulline. This will be used to determine both the identity of the fraction(s) that contain the amino acids as well as the % recovery. The most logical way of proceeding is to test a complete set of fractions for each column and use this information to test the appropriate fractions only from the other columns. It will be necessary to choose a volume from each fraction to use in the test. Since the amount of amino acid applied is known, this appropriate volume can be calculated.

Growth tests

Preparation of solid medium(slants)

This procedure can be done in the same class meeting as the calibration of the columns.

In order to do this, you will need

1. a 50X solution of Vogel's salts
2. sucrose
3. agar
4. bacto caps and 13 x 100 mm test tubes
5. disposable 10 ml pipette
6. hot plate
7. autoclave

1. In a 250 ml Erlenmeyer flask, prepare 150 ml of a Vogel's minimal medium(VM). This contains Vogel's salts (at 1 x) which is taken from a 50 X solution and 1.5% sucrose. Agar is added to a concentration of 2 %. After adding the agar, heat the solution on a stirring hot plate to melt the agar. It is necessary to calculate the masses of agar and sucrose that will be used as well as the volume of the 50 X Vogel's salts.

2. When the agar has dissolved, transfer 25 ml to three separate flasks. Remove a 50 ml aliquot and transfer to another flask. These portions will be supplemented with the various amino acids.

3. For three of the 25 ml portions and the 50 ml, portions add the appropriate amino acid to a 1 mM concentration. Arginine is added to the 50 ml portion while glutamate, citrulline and ornithine are added to the 25 ml portions. The amino acids are taken from a 10 mM stock solution that will be provided and you need to figure out what volume is used. Make sure to label the flasks appropriately. The last 25 ml portion will contain the unsupplemented medium.

4. Stir and heat the solutions on a hot plate to dissolve the agar.

6. Prepare the slants. When the agar has completely dissolved, take a disposable 10 ml graduated pipette and remove 10 ml. Dispense 2 ml each into 10 clean 13 x 100 mm test tubes. The agar will solidify when it cools and it is possible that the agar will solidify in the pipette. If this happens, re submerge the tip of the pipette in the heated solution.

7. Repeat with all of the solutions and prepare for sterilization. Cap the tubes with small white plastic caps (called Bacto caps) and label the tubes appropriately. Use a water insoluble sharpie pen to write on the glass or else use labels.

8. Sterilize the slants. This is done by using the autoclave which heats the slants at high pressure. At the end of the cycle, the slants are removed when still hot and allowed to cool at an angle and thus form an uneven surface or slant in the test tube. An easy way to do this is to allow the test tube rack to lean against a book as it cools.

12. Once the slants have cooled, store them at 30 °C overnight. If they have been properly sterilized, there should be no growth.

13. Store them at 4 °C. You will inoculate them with the various mutants in the next class.

Inoculation of the solid medium(slants)

In order to do the inoculation of the slants, you will need the following

1. stocks of the six unknowns
2. slants of VM, VM+ Glu, VM + Cit, VM+ Orn and VM+ Arg that were previously prepared.
3. 95% ethanol for sterilization
4. inoculating needle
5. Bunsen burner.

1. The slants were stored at 30 °C overnight to check for contamination. Following that, the slants are stored in the cold room. Remove the slants from the cold room

2. Sterilize your working area .In order to do this, make sure you are working in an area that is relatively free from contamination. There should be a smooth tabletop that is sterilized by spraying with ethanol and wiping. A lit Bunsen burner should be kept nearby.

3. Obtain a set of mutants from the instructor. These have been prepared for you and are labeled as A through F. Each mutant will be tested for growth on all types of prepared slants.

4. Using sterile technique, inoculate the slants. This will be demonstrated for you by the instructor. Every unknown must be inoculated once on VM, VM+ Glu, VM+Cit and VM +Orn and twice on VM+Arg

5. When all of the slants have been inoculated, place them in the 30 °C oven and store for two days. Then remove and store at room temperature where they will receive regular amounts of light. If they cannot be stored by a window, a lamp can be used with a timer to simulate daylight.

6. You should check for growth after a total of 4 days and make a chart that records each type of medium with each strain and where growth did and did not occur. Your chart should look like the one shown below

7. You can store these on the top of the bench. You will need them for subsequent experiments. This represents the end of the growth test portion of the project.

Strain	Growth on medium supplemented with					
	Nothing	Glutamate	Citrulline	Ornithine	Arginine	arginine
A						
B						
C						
D						
E						
F						

Measuring levels of intermediates

Instructions for preparing liquid medium

1. You will prepare a solution a 600 ml solution of Vogel's minimal (1 X) with 1.5% sucrose and 0.2 mM arginine. The Vogel's is taken from a 50 X solution and take the arginine from a 10 mM solution. You will need to calculate the appropriate volumes for these as well as the weight of sucrose used. Dissolve the sucrose using a stirring plate.
2. Divide the solution into 6 100 ml portion and transfer to 250 ml Erlenmeyer flasks. Label and cap with a foam plug. In order to sterilize the solutions, autoclave them as directed by the instructor.
3. Inoculate the medium. This will be done as demonstrated by the instructor. In order to do this, 5 ml of sterile water is added to one of the VM +Arg test tubes from the growth tests(all of the mutants grew in Arg). Shake to suspend the conidia in the water and allow a few minutes to settle. Then, using sterile technique, add the 5 ml to the Erlenmeyer flask which contains the liquid medium as shown by the instructor. The slant should be disposed of properly by autoclaving. The mutants should be allowed to grow from 12 to 24 hours on a shaker and thus, it is necessary to inoculate the day before.
4. Grow from 12 to 24 hours on a shaker at room temperature.

Measuring pools of arginine, ornithine and citrulline

1. Prior to this lab. someone should have inoculated the liquid medium with the various mutants and there should be copious amounts of mycelia in the flasks. If this is not the case, it might be necessary to grow the flasks for a longer period of time or repeat the experiment. _
2. For each of the mutants, collect the mycelia by filtration on a Buchner funnel. Use Whatman filter paper 2 (ask the instructor). Wash the mycelia pad with 2 x 10 ml aliquots of distilled water.
3. Transfer the mycelia to a 15 ml conical centrifuge tube that contains 3.00 ml of distilled water. It should be possible to simply peel the mycelia off the filter paper and no filter paper should be transferred to the conical tube.
4. Place the tube in a rack in a boiling water bath for 15 minutes.
5. Remove the tubes and allow them to cool to room temperature.
6. Centrifuge the tubes carefully in a table top centrifuge at low speed for 5 minutes
7. Carefully remove 1.0 ml of the supernatant. It is better to leave some of the

liquid behind rather than removing some of the precipitated material. This liquid contains the pools of the amino acids as well as all the other soluble metabolites. Add 1.0 ml of 0.116 M citrate buffer, pH 5.3. This sample is now ready to be applied to the cation exchange column and the standard elution schedule is followed. Those fractions that contain arginine, ornithine and citrulline are collected and assayed by the colorimetric tests (Sakaguchi, Chinard and Koritz Cohen respectively).

8- Decant the remaining liquid from the pellet and add 3.0 ml of 0.5 M NaOH (this solution will be provided for you). Cover the tube with parafilm, label appropriately and store at room temperature for at least 5 days. Measure the protein using the Lowry assay.

Sakaguchi Test for Arginine

Reference: *J. Biol. Chem.*, **222**, (1956), 225.

Solutions

1. 100 ml of 20 mg/ml thymine in 10% NaOH.(MW of thymine=126.1g/mole and MW of NaOH=40g/mole)
2. 100 ml of 0.04% 1-naphthol in 95% ethanol(MW of naphthol=144.17g/mole)
3. 100 ml of 2% sodium thiosulfate(MW=158.1g/mole)
4. 1 :5 solution of bleach.
5. 100 ml of 1 mM arginine(MW=174.20 g/mole)

Prior to the assay, mix 1 part solution 1 and 1 part solution 2.

Assay instructions

1. Label a set of 13 x 100 mm test tubes.
2. Add the standards and samples to test tubes in an appropriate volume. The standard curve contains:
Standards: 0,50, 100, 150, 200 and 250 nmoles of arginine
Samples: pick an appropriate volume up to 2.0 ml
You need to calculate the volumes needed for the standards.
3. If the sample is an aqueous solution, add 0.2 M NaOH to a volume of 2.0 ml. If the sample is in 0.2 M NaOH, add water to a volume of 2.0 ml.
4. Add 0.5 ml of naphthol-thymine mixture. Mix well.
5. Add 0.2 ml of the bleach solution. Mix well immediately.
6. After exactly one minute, add 0.2 ml of thiosulfate solution. Mix well.
7. Read the absorbance at 500 nm.

Notes and hints on the assay.

It is important that the tubes and be cold and that there be exactly 60 seconds between the addition of the bleach and the thiosulfate. The best way to do this is to stagger the samples. Add bleach to the first tube at 0 seconds and to the second at 15 seconds, the third at 30 sec, the fourth at 45 seconds and at 60 seconds start add the thiosulfate.

Koritz-Cohen Test for Citrulline

Reference: *J. Biological Chemistry*, 209, (1954), page 145

Solutions

1. 500 ml of 50% H₂SO₄
2. 100 ml of 3% diacetylmonoxime (2,3 butanedione)(MW=101.1 g/mole)
3. 100 ml of 0.4% p-diphenylamine sulfonate(MW=271.3g/mole)
4. 100 ml of 1 mM L-citrulline(MW=175.2g/mole)
5. 100 ml of 1 % K₂S₂O₈(potassium persulfate)(MW=270.32g/mole)
6. 100 ml of 1 M HCl (made from 12.0 M HCl)

Solutions that have to be prepared using these reagents:

Make a mixture that contains 4.0 ml of 50% H₂SO₄, 0.1 ml of 1 M HCl, 0.2 ml of 3 % diacetylmonoxime and 0.2 ml of 0.4% p-diphenylamine sulfonate per sample.

Assay instructions:

1. Label 18 x 150 mm test tubes
2. Add the standards or the samples. The samples have a volume of 2.0 ml or less. The standards contain the following amounts of citrulline: standards: 0, 50, 100, 150,200 and 250 nmole. You will need to calculate what volume of 1 mM citrulline is necessary to make these standards.
3. Bring the volume up to 2.0 ml with distilled water.
4. Add 4.5 ml of the prepared mixture to each tube. Mix, cap with a white Bacto cap and place in a boiling water bath for 10 minutes.
5. Allow the tubes to cool until you can handle them.
6. Add 0.2 ml of 1% potassium persulfate
7. Mix and boil exactly one minute.
8. Allow the tubes to cool. Measure the absorbance at 540 nm.

Notes on this assay:

1. The colored compound produced in this assay is somewhat light sensitive. If it is left in the light for too long, it will break down. For this reason, read the absorbance values soon after the tubes have cooled.
2. This assay requires extremely acidic conditions. Be careful when handling the acidic solutions. Use gloves and make sure that you clean up all spills.

Chinard Test for Ornithine

Reference: *J. Biological Chemistry*, 199, (1952),. page 91

Solutions

1. 100 ml of 6 M H_3PO_4 (from 18.0 M)
2. A mixture containing 25 mg of ninhydrin, 0.4 ml of 6 M H_3PO_4 and 1.6 ml of glacial acetic acid per sample. This is already prepared
3. 1 mM ornithine

Assay instructions:

1. Label 13 x 100 mm test tubes.
2. Add standards and samples to the test tubes. The samples must have a volume less than or equal to 1.0 ml. For the standards use volumes equivalent to: 0, 50., 100, 150, 200, and 250 nmole.
You will have to calculate what volume is needed from the 1 mM standard to give these amounts of ornithine.
3. Bring the volume up to 1.0 ml with distilled water.
4. Add 2.0 ml of the prepared mixture.
5. Mix, cap, and place in a boiling water bath for 60 minutes.
6. Allow the tubes to cool to room temperature. Read the absorbance at 500 nm.

Notes on the assay:

- 1.) This assay calls for use of strong acids. Use caution: you might want to use gloves and make sure that you clean up all spills.
- 2.) Ninhydrin reacts with all free amine groups so it will react with proteins, including those in your skin. You might notice purple spots on your hands and fingers if you did not use gloves. The color is due to the ninhydrin and it will fade in a few days.

Lowry Test for Protein

Reference: *J. Biological Chemistry*, 193, (1951), page 265

Solutions

1. 100 ml of 2.68% potassium sodium tartrate(MW=282.22g/mole)
2. 100 ml of 1% $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ (MW=249.68g/mole)
3. 1.0 L of 2% Na_2CO_3 in 0.1 M NaOH(MW of Na_2CO_3 =106g/mole and MW of NaOH=40 g/mole)
4. 200 ml of 1.0 M Folin reagent(from 2.0 M Folin)
5. 100 ml of 1 mg/ml BSA (Bovine Serum Albumin)

To be prepared on the day of the assay:

Make a mixture that is: 1 part 2.68 % potassium sodium tartrate, 1 part Copper (II)sulfate pentahydrate and 100 parts sodium carbonate in NaOH solution.

Assay instructions:

1. Label 13 x 100 mm test tubes.
2. Add samples and standards. Add a volume of BSA standard that contains the following:
standards: 0, 100, 150, 200, 250 and 300 g BSA
samples: add a volume of 1.2 ml or less
3. Bring the total volume to 1.2 ml with distilled water
4. Add 3.0 ml of the tartrate: copper (II) sulfate: Na_2CO_3 mixture. Mix immediately and let stand for 10 min.
5. Add 0.3 ml of Folin reagent and mix immediately with vortex. Let stand for 30 min and then measure the absorbance on the UV-VIS spectrophotometer at 720 nm.

