

CLS 281

Basic Biochemistry and Biomolecules

جامعة
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Experiment 2

Color Tests for Proteins and Amino Acids

(Test for Specific Amino Acids)

Review

All are colorimetric test.

Lecture 1: General Color Tests for Proteins

Detection of protein in a sample (generally without specifying one amino acid).

Lecture 2: Specific Color Tests for Proteins and Amino Acids

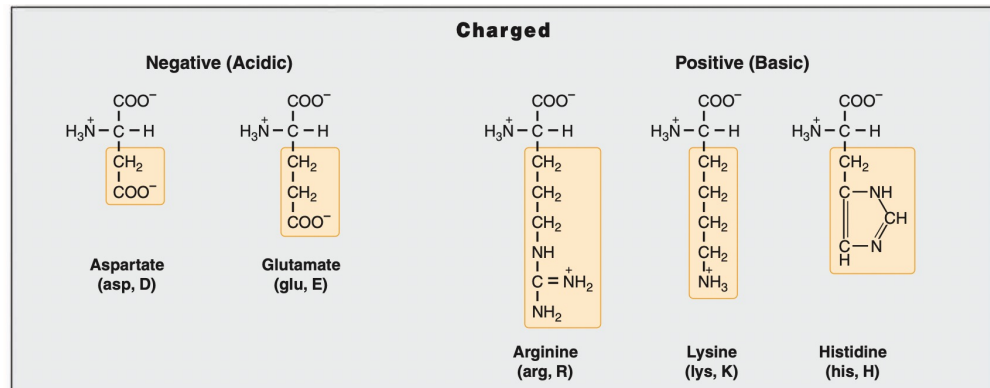
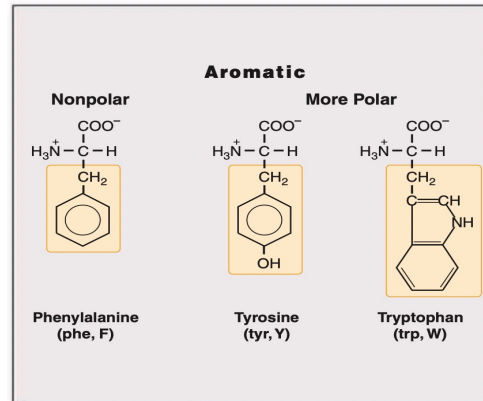
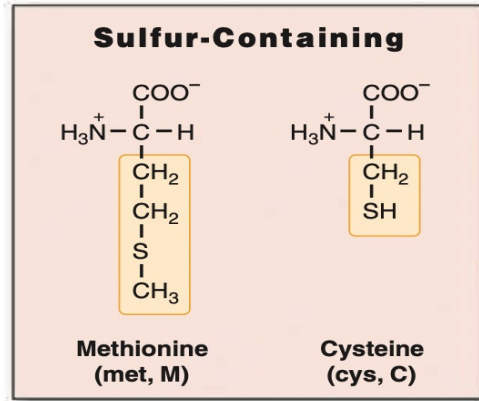
Each test detects one specific amino acid.

Overview

Test for specific amino acids:

1. *Millon's Test*
 2. *Hopkins-Cole Test*
 3. *Sakaguchi Test*
 4. *Lead Acetate (Lead sulfide) Test*
 5. *Folin's Test*
- All the experiments in this lab are used for the detection of **specific amino acids**, but they're not specific for protein as each test also detects specific groups present in **other organic compounds as well**.

Highlight of some Amino Acids



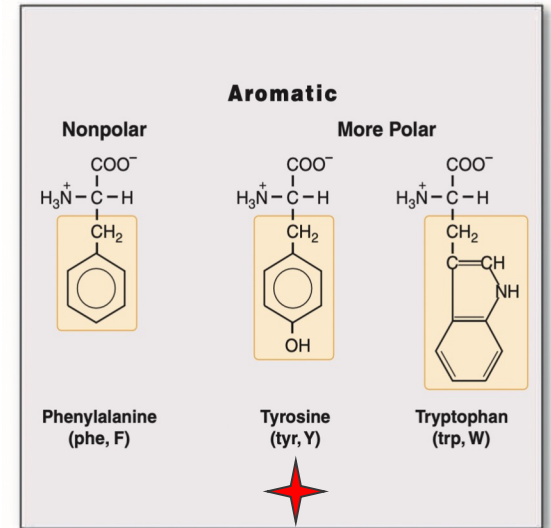
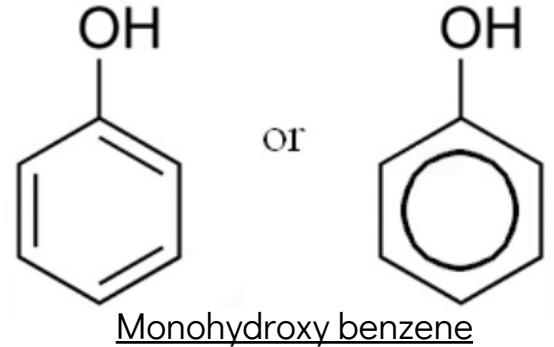
01 *Millon's Test*

- **Aim**

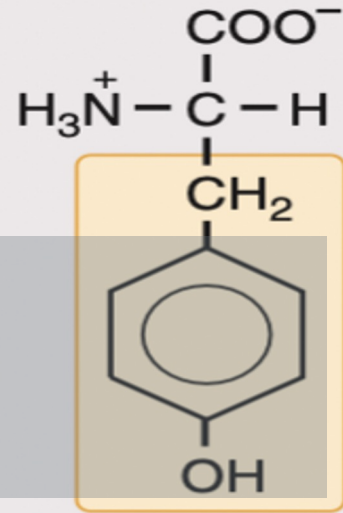
Millon's test is used for detecting the presence of monohydroxy benzene derivatives (e.g: Tyrosine, Tyrosine derivatives, Phenol).

- **Specificity**

- Millon's test is a specific test for tyrosine, but it is not a specific test for protein as it also detects the phenolic group present in other compounds as well.
- Therefore, while performing Millon's test, it is essential that other tests like the Biuret test and Ninhydrin test also be performed.



Tyrosine is the
only amino acid
containing the
phenol group.



Tyrosine
(tyr, Y)

01

Millon's Test Principle

- **Reagent**

Millon's reagent is a solution of mercuric and mercurous ions in nitric and nitrous acids.

- **Principle**

The phenolic group of tyrosine reacts with Millon's reagent and gives a red color, which is due to the nitration of the phenol group in tyrosine by the nitric acid present in the reagent, followed by the combination of nitrated tyrosine with the mercury ions in the solution.

Reaction step:

1- Monohydroxy benzene + Nitric acid \rightarrow Nitrated Monohydroxy benzene

2- Nitrated Monohydroxy benzene + mercury ions \rightarrow mercury salt (Red color)

01

Millon's Test Procedure

Note: swirl the samples and reagent bottles before use.

Steps	Tube No.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	
1	Sample	0.02% phenol	0.02% phenylalanine	1% gelatin	0.02% tyrosine	0.02% salicylic acid	1% egg albumin	H ₂ O	
	Volume	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml	
2	Reagent	Millon's Reagent							
	Volume	3-5 drops	3-5 drops	3-5 drops	3-5 drops	3-5 drops	3-5 drops	3-5 drops	

3- Incubate in a boiling water bath for 2 min.

4- Note the color formed

Samples:

1. Phenol
2. **Phenylalanine:** amino acid.
3. **Gelatin:** is a translucent, colorless, flavorless solid substance derived from collagen. It contains tyrosine
4. Tyrosine
5. **Salicylic acid:** is a monohydroxy benzoic acid, a type of phenolic acid.
6. **Egg albumin:** protein contains tyrosine
7. Water

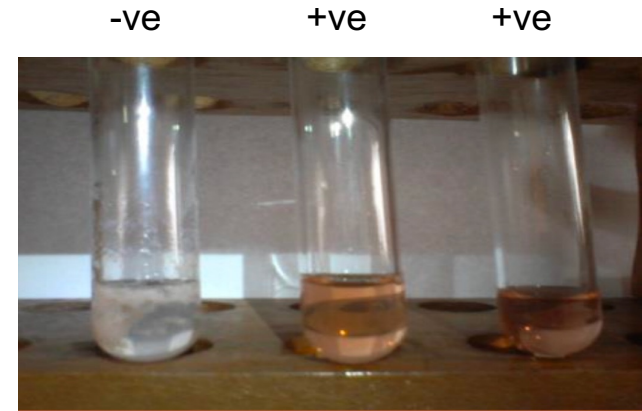
01 *Millon's Test Result*

- **Positive result >>> light pink to red**

This indicates the **presence** of tyrosine or tyrosine-containing protein or phenolic compounds.

- **Negative result >>> no change in color or giving different colors (but not red).**

This indicates the **absence** of tyrosine or tyrosine-containing protein or phenolic compounds.



Millon's Negative Test

Absence of tyrosine
or phenol-containing
compounds

Red or pink colored
precipitate absent



Millon's Positive Test

Presence of tyrosine
or phenol-containing
compounds

Red or pink colored
precipitate present

02 Hopkins-Cole Test

- Aim

Hopkin's Cole test is a specific test used for the detection of **indole ring** that is found in the **tryptophan** amino acid, which in turn helps in the identification of proteins containing tryptophan.

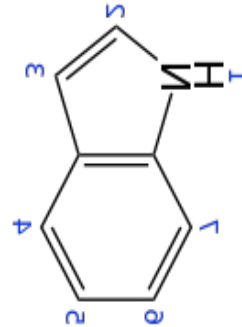
- Reagent

1. The Hopkins-Cole reagent contains Glyoxylic acid.

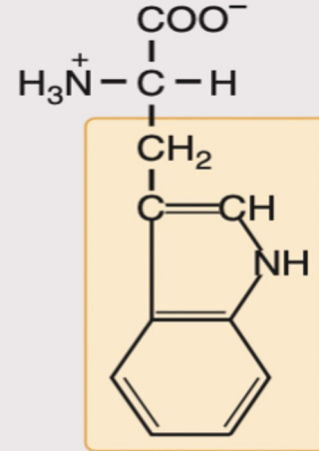
Hopkins-Cole reagent preparation:

- Magnesium powder + Oxalic acid → Magnesium glyoxylate.
- Magnesium glyoxylate + Acetic acid → Glyoxylic acid.

2. Concentrated sulfuric acid H₂SO₄



Indole



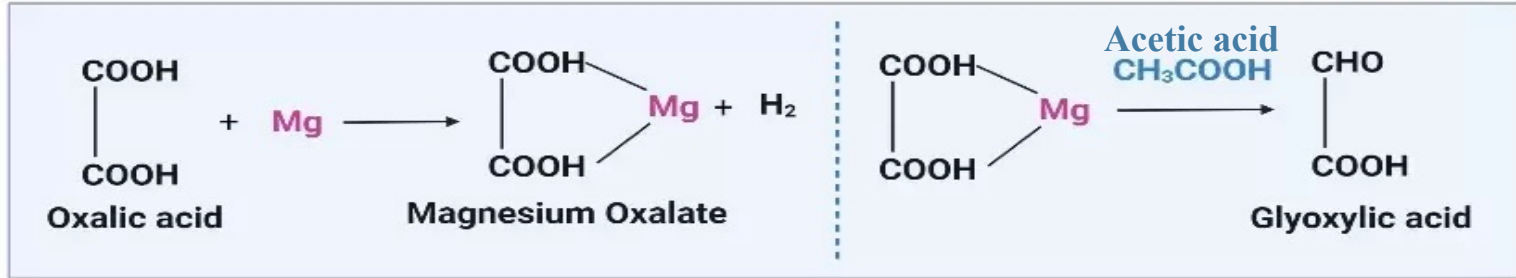
Tryptophan
(trp, W)

02

Hopkins-Cole Principle

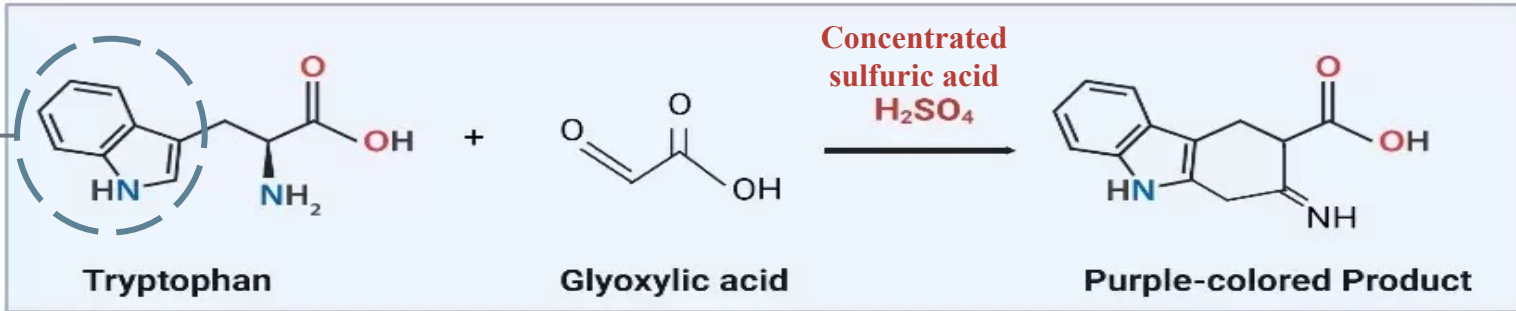
Hopkins-Cole reagent preparation:

1)



2)

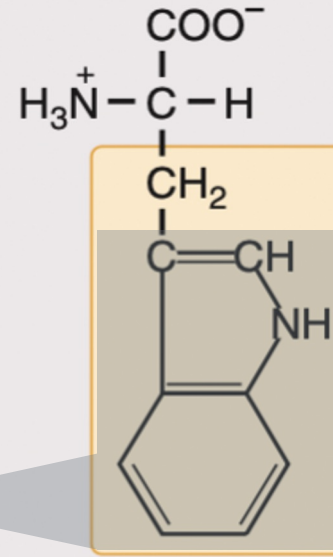
Indole ring



The **purple ring** is formed by the reaction of glyoxylic acid (CHO- COOH) with the indole ring of tryptophan in the presence of sulfuric acid (H₂SO₄)


Tryptophan is the
only amino acid
containing the
Indole nucleus.

The indole nucleus of Tryptophan is responsible for the **violet ring** found at the junction between the two layers in this reaction.



Tryptophan
(trp, W)

02 Hopkins-Cole Test Procedure

Steps	Tube No.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
1	Sample	0.02% tyrosine	0.02% tryptophan	1% casein	1% gelatin	1% egg albumin	H ₂ O
	Volume	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
2	Reagent	Hopkins-Cole Reagent					
	Volume	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml
3	Reagent	 <p>*Concentrated H₂SO₄ (Note: The opening of the tube must point away from the face).</p>					
	Volume	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml

Samples:

- Tyrosine
- Tryptophan
- Casein: protein contains tryptophan
- Gelatin: incomplete protein because it lacks the essential amino acid tryptophan
- Egg albumin: protein contains tryptophan
- Water: as a negative control



- Add H₂SO₄ slowly to the wall of the tube that is held at a slanting angle so that the two liquids form separate layers.
 - Observe the color at the zone of contact of the two fluids. If no color appears, gently rotate the tube to develop the colored ring but do not mix.

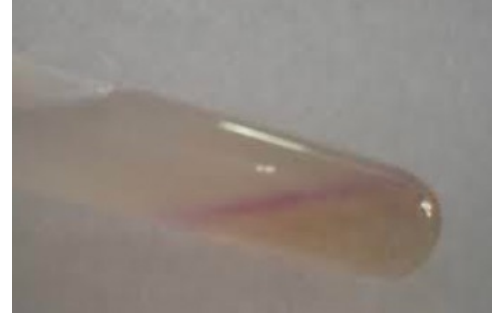
02 Hopkins-Cole Test Result

- Positive result >>> formation of a **purple-colored ring** at the junction of two layers.

This indicates the presence of tryptophan-containing proteins.

- Negative result >>> absence of a purple-colored ring in the test tube

This indicates the absence of tryptophan-containing proteins.



03 Sakaguchi Test

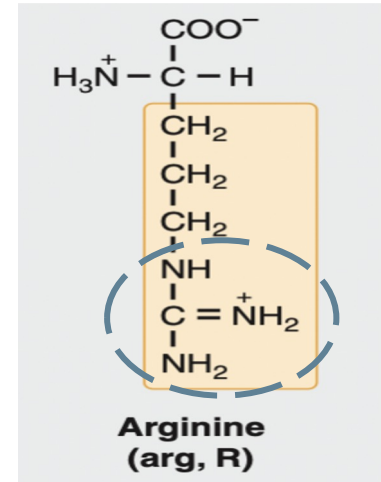
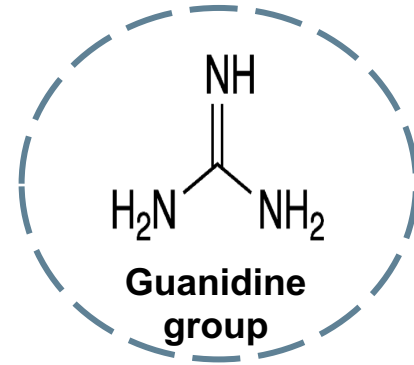
- Aim

This test is specific for the guanidine group of arginine $\text{HNC}(\text{NH}_2)_2$, but a non-amino acid, like creatine which contains this group, will also answer this test.

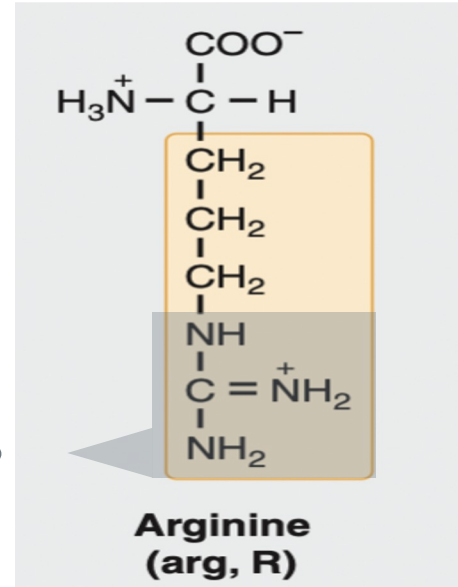
- This test is positive for all proteins containing arginine and arginine amino acid itself.

- Sakaguchi reagent

- It consists of sodium hypobromite and 1-naphthol.

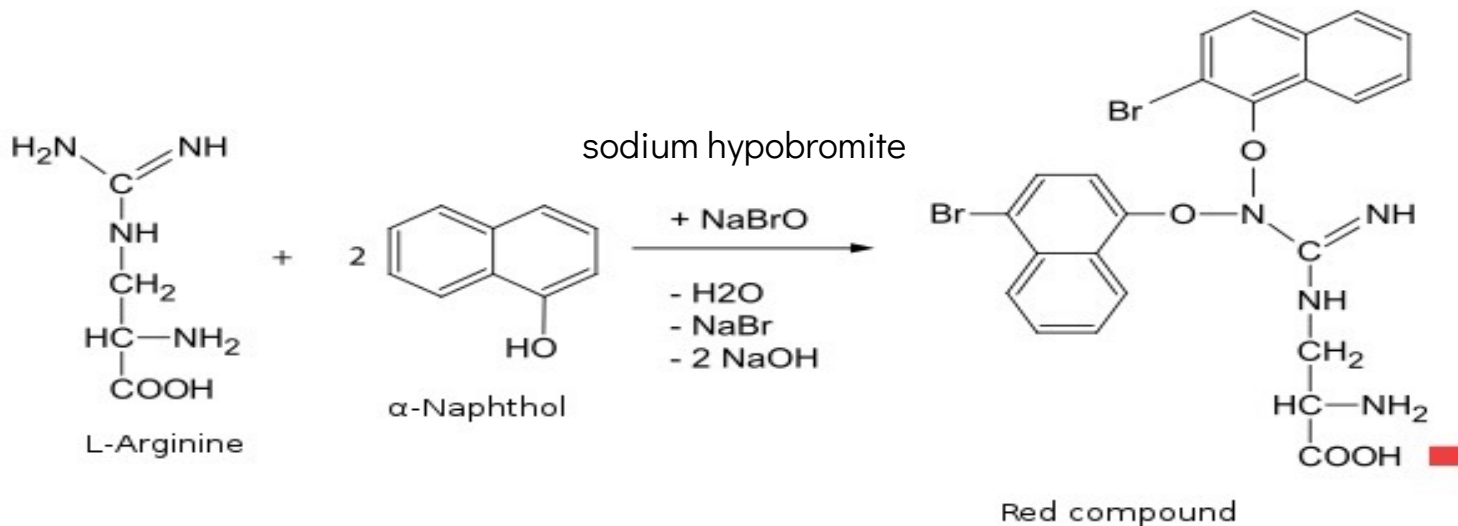


Arginine is the
only amino acid
containing the
Guanidine group.



03

Sakaguchi Test Principle



- When arginine reacts with α -Naphthol and Sodium hypobromite (NaOBr), a **red color** results.
- The **red color** is due to a reaction between the hypobromite and the -NH₂ group of the guanidino part of arginine.

03

Sakaguchi Test Procedure

Steps	Tube No.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
1	Sample	0.02% urea	0.02% creatine	0.02% arginine	0.1% gelatin	H ₂ O
	Volume	5 ml	5 ml	5 ml	5 ml	5 ml
2	Alkaline Reagent	10% NaOH				
	Volume	1 ml	1 ml	1 ml	1 ml	1 ml
3	Reagent	0.02% α -Naphthol				
	Volume	1 ml	1 ml	1 ml	1 ml	1 ml
4	Mix, and incubate at room temperature (RT) for 3 minutes					
5	NaOBr					
	4 drops	4 drops	4 drops	4 drops	4 drops	4 drops

Samples:

- Urea: is an organic compound
- Creatine: contains guanidine group
- Arginine
- Gelatin: contains arginine
- Water: as control

03

Sakaguchi Test Result

- Positive result >>> formation of **red color**.

This indicates the presence of an arginine or guanidinium compound.

- Negative result >>> absence of red color.

This indicates an absence of arginine or a guanidinium compound.



**Sakaguchi
Negative Test**

**Absence of arginine
or a guanidinium
compound**

**Red colored complex
absent**



**Sakaguchi
Positive Test**

**Presence of arginine
or a guanidinium
compound**

**Red colored complex
present**

04 *Lead Acetate Test*

- Lead Acetate or Lead sulfide test.

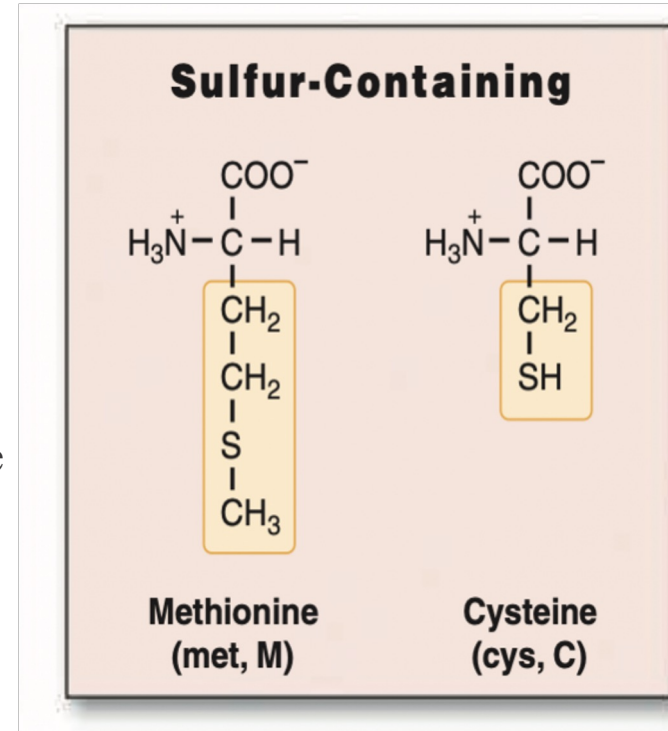
- Aim

Used for detecting the presence of **Sulfur group -S** in Cystine and free cysteine.

- **Cysteine** is an amino acid.
- **Cystine** is formed when two molecules of cysteine are joined together via a disulfide bond.

- Reagent

- Lead Acetate
- NaOH



04

Lead Acetate Test

- Proteins or peptides containing amino acids with Sulfur group heated with NaOH to split the sulfide group from amino acids.
- Then, lead acetate reacts with free sulfide ions resulting in the formation of lead sulfide (pbs) a brown-to-black color.



**Lead Sulfide
Negative Test**

**Black precipitate
absent**

**Sulfur-containing
amino acids absent
(cysteine or cystine)**



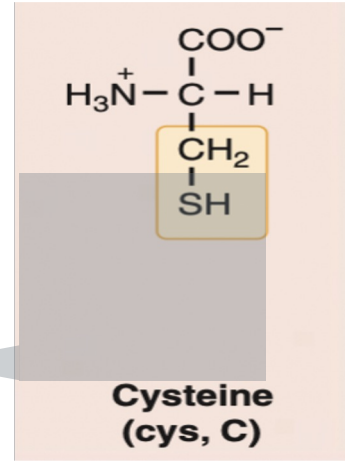
**Lead Sulfide
Positive Test**

**Black precipitate
present**

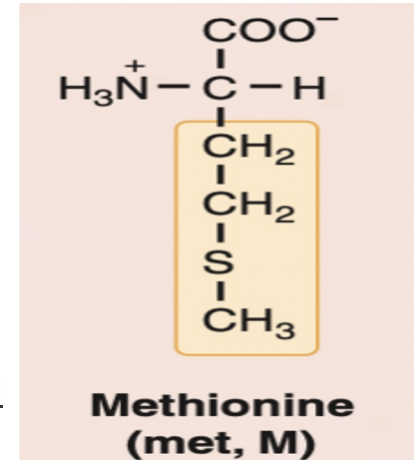
**Sulfur-containing
amino acids present
(cysteine or cystine)**

Cysteine amino acid containing the Sulfhydryl group

which is released by the treatment with NaOH.



Methionine doesn't give a positive result in this test as the sulfur in the thioester bond in methionine is **not** released by the treatment with NaOH.



04

Lead Acetate Test Procedure

Steps	Tube No.	Tube 1	Tube 2	Tube 3	Tube 4
1	Sample	0.02% methionine	0.02% cystine	1% egg albumin	H ₂ O
	Volume	2 ml	2 ml	2 ml	2 ml
2	Alkaline Reagent	5% NaOH			
	Volume	5 ml	5 ml	5 ml	5 ml
3	Reagent	Lead Acetate			
	Volume	Few crystals	Few crystals	Few crystals	Few crystals

4- Incubate in boiling water bath for 10 minutes with occasional mixing.
Describe the color changes in each test tubes.

05 Folin's Test

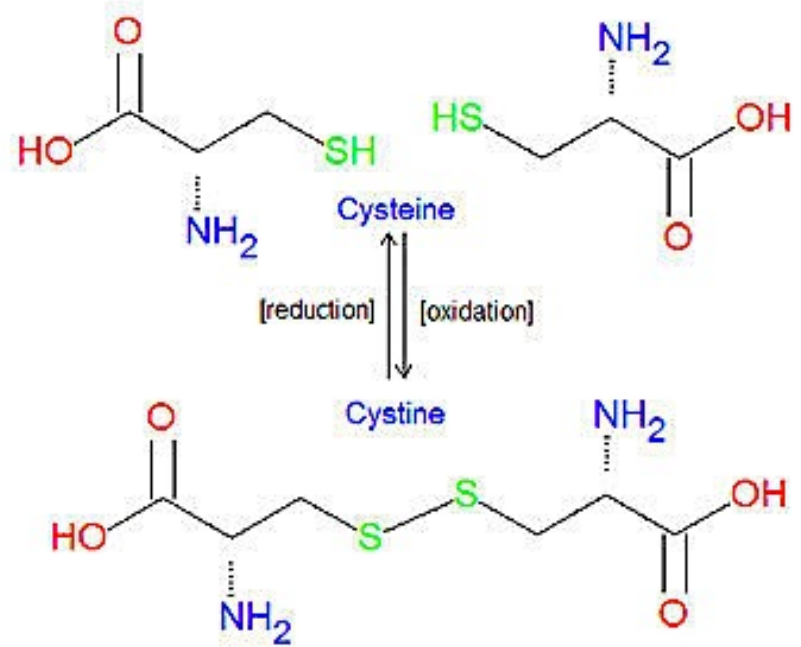
- Aim

This test is for the detection of free cystine.

- Reagent

1. sodium carbonate and sodium sulfite.
2. Folin's uric acid reagent (phosphotungstic acid)

- This test can be used for the quantitative determination of cystine.



05 *Folin's Test*

- Principle
 - Proteins or peptides containing cystine are first **hydrolyzed** by sodium carbonate and sodium sulfite to free the cystine.
 - This is followed by the addition of Folin's uric acid reagent (phosphotungstic acid) which is reduced to **tungsten blue.**
- Result
 - Positive: **Tungsten blue.**

05

Folin's Test Procedure

Steps	Tube No.	Tube 1	Tube 2	Tube 3	Tube 4
1	Sample	0.1% methionine	1% egg albumin	0.1% cystine	H ₂ O
	Volume	1 ml	1 ml	1 ml	1 ml
2	Reagent 1	Sodium Carbonate (saturated)			
	Volume	7 ml	7 ml	7 ml	7 ml
3	Reagent 2	20% Sodium Sulfite			
	Volume	3 ml	3 ml	3 ml	3 ml
4	Incubate at R.T for 5 min.				
5	Reagent 3	Uric acid reagent			
	Volume	1 ml	1 ml	1 ml	1 ml

Note the color formed

Summary of test for specific amino acids:

Test	Detect	Principle	Positive Result	Negative Result
Millon's Test	Monohydroxy benzene derivatives (e.g: Tyrosine, Tyrosine derivatives, Phenol).	Nitrification of the phenol group in tyrosine by the nitric acid followed by the combination of nitrated tyrosine with the mercury ions in the solution.	Red	Colorless
Hopkins-Cole Test	Indole ring found in the tryptophan amino acid.	Glyoxylic acid reacts with the indole ring of tryptophan in the presence of sulfuric acid (H ₂ SO ₄), forming a purple ring.	Purple ring	Colorless
Sakaguchi Test	Guanidine group in arginine.	The reaction of the NH ₂ group of the guanidino part of arginine reacts with α -Naphthol and Sodium hypobromite forming a red color complex.	Red	Colorless

Summary of test for specific amino acids:

Test	Detect	Principle	Positive Result	Negative Result
Lead Acetate (Lead sulfide) Test	Sulfhydryl group in Cystine, free cysteine.	Proteins or peptides containing amino acids with Sulfur group heated with NaOH to split the sulfide group from amino acids. Then, lead acetate reacts with free sulfide ions resulting in the formation of lead sulfide (pbs) a brown-to-black color.	Black precipitate	Colorless
Folin's Test	<u>free cystine.</u>	Protein or peptide must first be hydrolyzed by sodium carbonate and sodium sulfite, followed by the reduction of Folin's uric acid reagent (phosphotungstic acid) to tungsten blue.	Tungsten blue.	Colorless

Guideline for writing the lab report

Total: 5 marks

All the following information should be included in your report:

- a) Course # (CLS 281)
- b) Experiment title
- c) Date of the experiment
- d) Student's names and university ID#
- e) Section #

The lab report is broken down into 6 sections:

1. Experiment **title**
2. The **aim** of the experiment (objective, or what the test detects specifically) (1 mark)
3. **Principle** (chemical reaction) (1 mark)
4. **Methodology** (written in **steps**, **not in tables**)
5. **Result** (1 mark)
6. **Interpretation or Comment** (2 mark)

Deadline: Next lab Submission: via email