Reading material for 2nd year LS2102 Biochemistry practical prepared by Dr. Rituparna Sinha Roy. Distribution of this teaching material is not permitted.

Reading material was prepared from three books and internet links.

1. Laboratory Handbook on Biochemistry (Authors: S. Shanmugan, T. Sathish Kumar, K. PanneerSelvam)
2. An Introduction to Practical Biochemistry (Author: David T Plummer)
3. Introductory Practical Biochemistry (Authors:S. K. Sawhney, R. Singh)
4. Internet sources and wikipedia

Qualitative Test of amino acids

Introduction

The food we consume can be divided in three portions. They are carbohydrates, which is our body's energy source, lipids, the body's energy reserve and proteins. Proteins are large polymeric molecules made up of amino acids synthesized by cells. In humans, twenty different amino acids are found, of which eight are known as essential amino acids since these cannot be synthesized by human cells. The amino acids contain an amino group (–NH_2) separated from a carboxylic acid group (–COOH) by a single C_α carbon. These amino acids are distinguished by the chemistry of the side chains attached to the C_α carbon. Thus depending on the nature of the side chain of the amino acids, they can be hydrophobic (water reluctant) or hydrophilic (Water fond). Hydrophilic amino acids are polar and may be charged under physiological pH.

Amino acids can be presented in both the ways as charged or neutral formula. Carboxylic acid groups (−CO_2H) can be deprotonated to become negative carboxylates (−CO_2^-), and α-amino groups (NH_2−) can be protonated to become positive α-ammonium groups ($^+NH_3$−). Generally, below pH 2.2, the predominant form will have a neutral carboxylic acid group and a positive α-ammonium ion (net charge +1), and above pH 9.4, a negative carboxylate and neutral α-amino group (net charge −1). The side chain is represented by "R", R = alkyl, aryl, thio group.

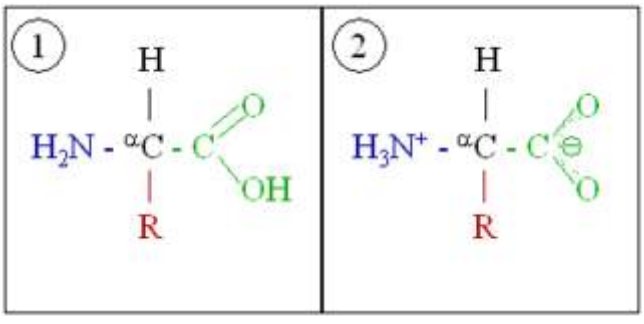


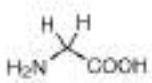
Figure 1.Generic structure of Amino acid in its (1) unionized and (2) Zwitterionic forms.

The 20 amino acids can be classified on the basis of their side chains:

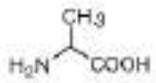
- Non-polar
- Polar-uncharged at physiological pH
- Polar-charged

Figure 2: Chemical formulae of the amino acids. Figure shows 3-letter code and one-letter code of amino acids

Small

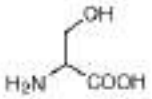


Glycine (Gly, G)
MW: 57.05

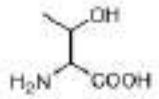


Alanine (Ala, A)
MW: 71.09

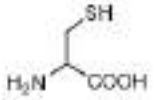
Nucleophilic



Serine (Ser, S)
MW: 87.08, pK_a ~ 16

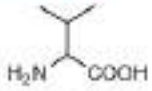


Threonine (Thr, T)
MW: 101.11, pK_a ~ 16

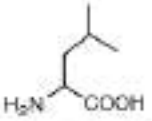


Cysteine (Cys, C)
MW: 103.15, pK_a = 8.35

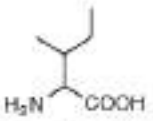
Hydrophobic



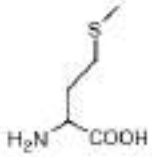
Valine (Val, V)
MW: 99.14



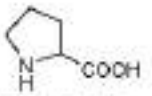
Leucine (Leu, L)
MW: 113.16



Isoleucine (Ile, I)
MW: 113.16

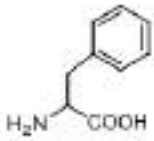


Methionine (Met, M)
MW: 131.19

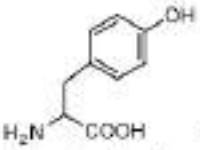


Proline (Pro, P)
MW: 97.12

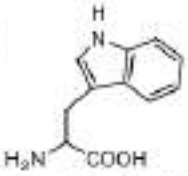
Aromatic



Phenylalanine (Phe, F)
MW: 147.18

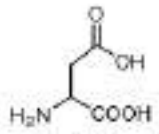


Tyrosine (Tyr, Y)
MW: 163.18

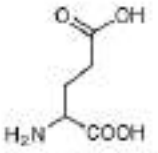


Tryptophan (Trp, W)
MW: 186.21

Acidic

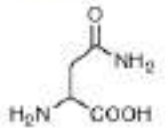


Aspartic Acid (Asp, D)
MW: 115.09, pK_a = 3.9

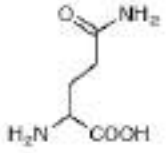


Glutamic Acid (Glu, E)
MW: 129.12, pK_a = 4.07

Amide

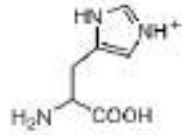


Asparagine (Asn, N)
MW: 114.11

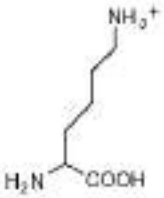


Glutamine (Gln, Q)
MW: 128.14

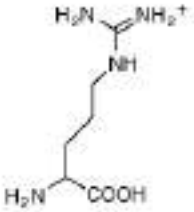
Basic



Histidine (His, H)
MW: 137.14, pK_a = 6.04



Lysine (Lys, K)
MW: 128.17, pK_a = 10.79



Arginine (Arg, R)
MW: 156.19, pK_a = 12.48

Table 1: The pKa values are given below for the 20 α-amino acids.

Source: *http://www.mhhe.com/physsci/chemistry/carey5e/Ch27/ch27-1-4-2.html*

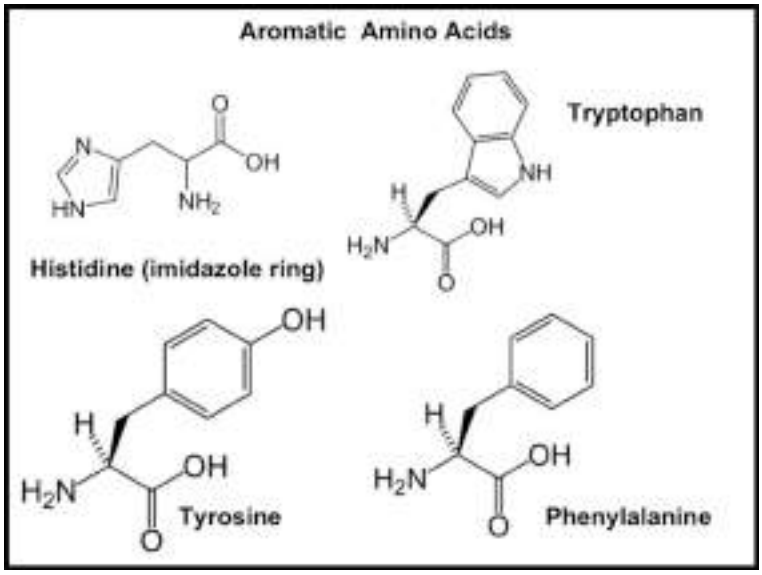
pKa_1= α-carboxyl group, pKa_2 = α-ammonium ion, and pKa_3 = side chain group.

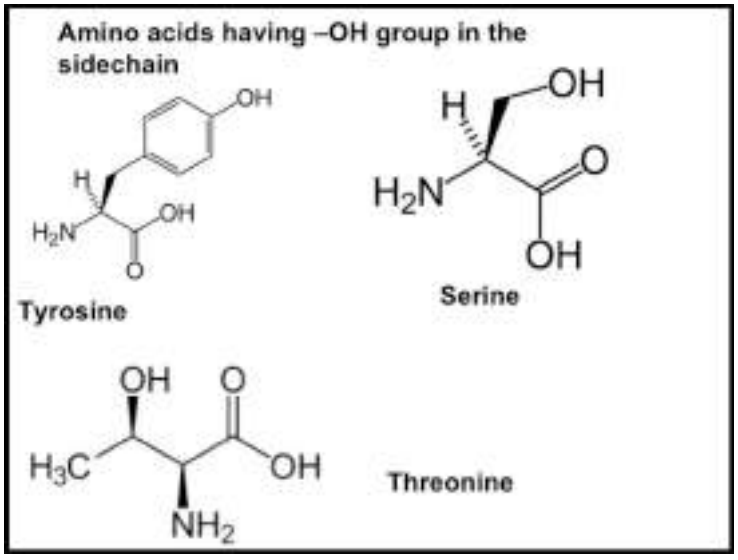
Amino acid	pKa_1	pKa_2	pKa_3
Glycine	2.34	9.60	---
Alanine	2.34	9.69	---
Valine	2.32	9.62	---
Leucine	2.36	9.60	---
Isoleucine	2.36	9.60	---
Methionine	2.28	9.21	---
Proline	1.99	10.60	---
Phenylalanine	1.83	9.13	---
Tryptophan	2.83	9.39	---
Asparagine	2.02	8.80	---
Glutamine	2.17	9.13	---
Serine	2.21	9.15	---
Threonine	2.09	9.10	---
Tyrosine	2.20	9.11	---
Cysteine	1.96	8.18	---
Aspartic acid	1.88	9.60	3.65
Glutamic acid	2.19	9.67	4.25
Lysine	2.18	8.95	10.53
Arginine	2.17	9.04	12.48
Histidine	1.82	9.17	6.00

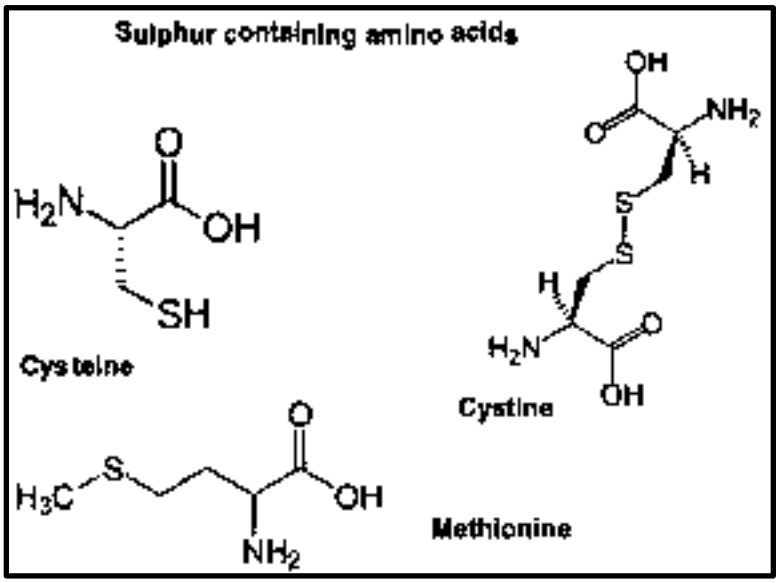
Isoelectric point (p*I*):(sometimes abbreviated to **IEP**)ThepI is the pH value at which the molecule carries no electrical charge or the negative and positive charges are equal.The zwitterions contain both positive and negative charges depending on the functional groups present in the molecule. For electrically neutral amino acids the p*I* can be calculated from the following equation.

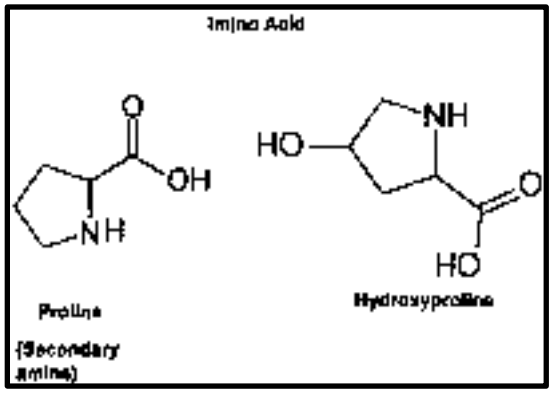
$$pI = \tfrac{1}{2}(pKa_1 + pKa_2)$$

In this practical we shall looking into the qualitative tests for amino acids, meaning how we can confirm the presence of a particular amino acid in the sample you have been given. Qualitative means quality wise i.e. color of the sample, solubility in different solvent, color change of a sample in presence of a particular test reagent.









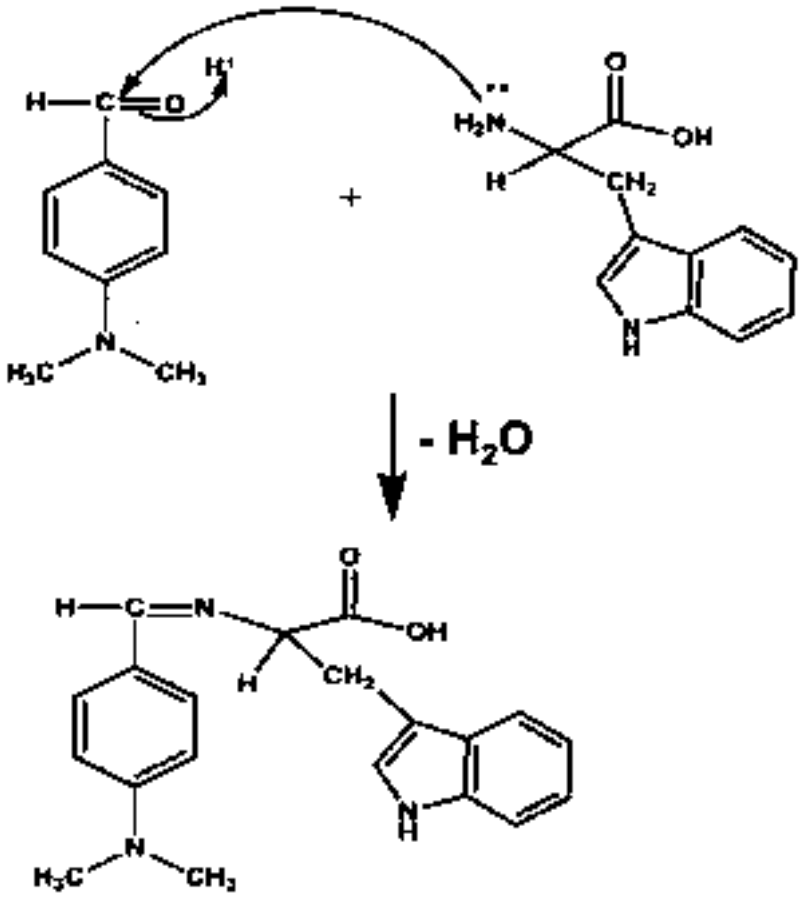
Principle of the method

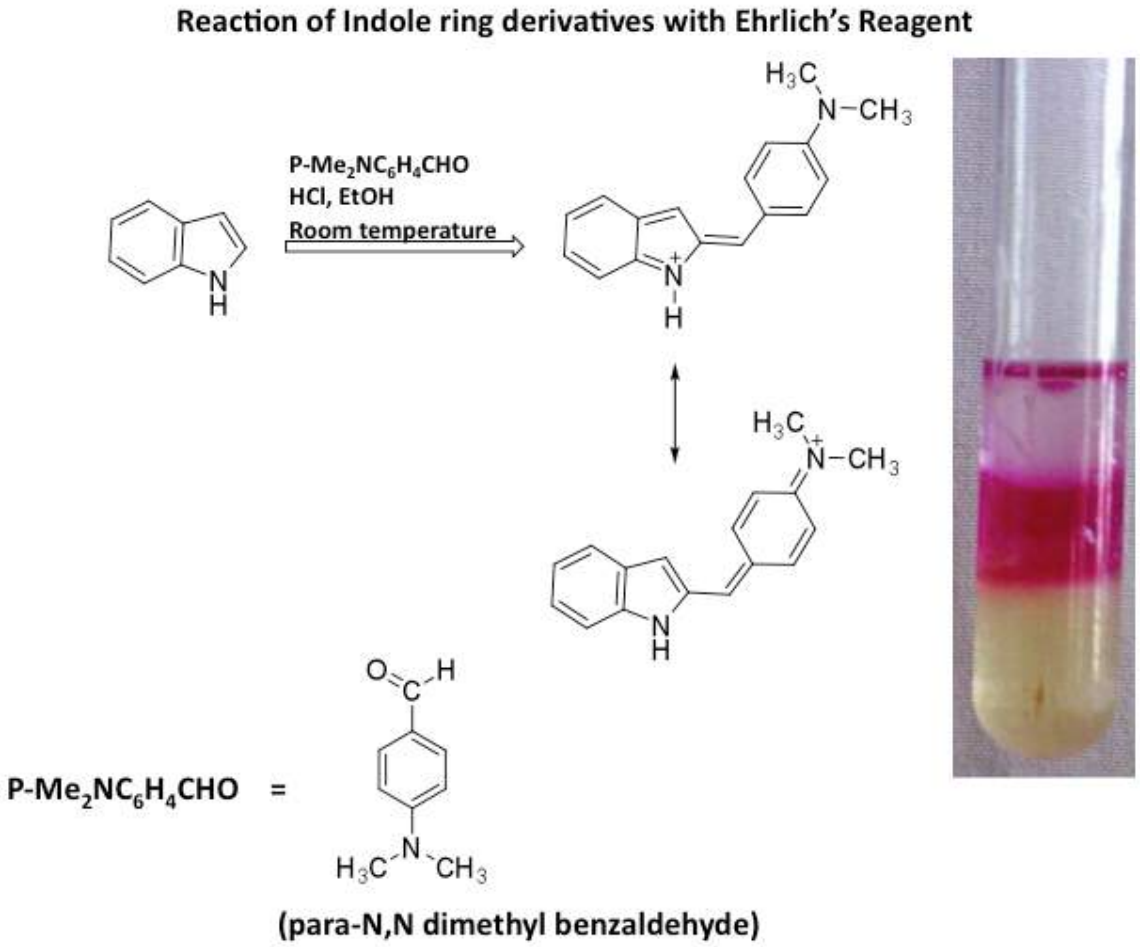
Colorimetry is a widely used tool in biochemistry. In proteins different functional groups chemistries react with small molecule probes to produce colored products. The intensity of these products can specify the functional groups present in the protein and in turn the protein concentration. The following assays are colorimetric assays for a qualitative determination of amino acids. You will use dyes to detect the presence of different functional groups in amino acids.

Erlisch test for Tryptophan:

The *p*-dimethylaminobenzaldehyde reacts with tryptophan to form a red colouredproduct .Initially it is **pink/red** and gradually it becomes **light purple blue**.

This Erlisch's reagent is prepared by dissolving 2.0g of *p*–dimethylaminobenzaldehyde (DMAB) in 50 mL of 95% ethanol and 50 mL of concentrated hydrochloric acid. It is best prepared fresh. The probable mechanism is given here:





Folins-Ciocalteu test for Tyrosine:

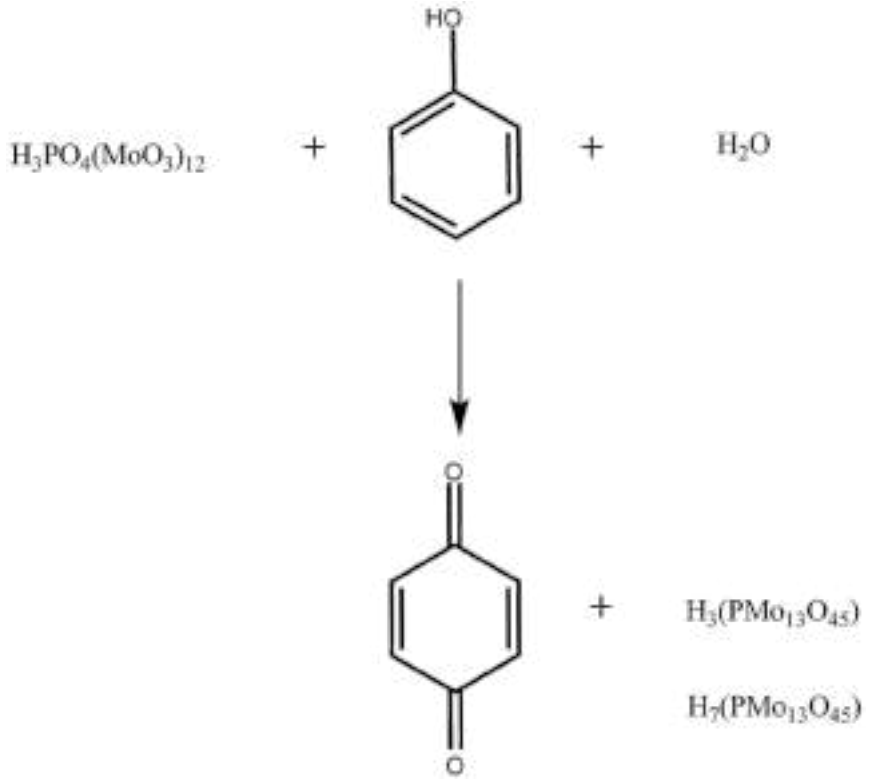
The phenolic group of tyrosine residue (amino acid) in a protein will produce a blue **purple** color complex, with λ_{max}in the region of **660 nm**wavelength, with Folin-Ciocalteu reagent which consists of sodium tungstate molybdate and phosphate.

This reagent is prepared by dissolving 10 g sodium tungstate and 2.5 g sodium molybdate in 70 mL water and thenaddition 5 mL 85% phosphoric acid followed by 10 mL concentrated hydrochloric acid. This mixture is refluxed for 10 h. Add 15 g lithium sulfate, 5 mL water and 1 drop bromine. Refluxedagain for 15min. Cool to room temperature and bring to 100 mL with water. Hexavalentphosphomolybdic/phosphotungstic acid complexes with the following structures are formed in solution.

$3H_2O•P_2O_5•13WO_3•5MoO_3•10H_2O$

$3H_2OxP_2O_5•14WO_3•4MoO_3•10H_2O$

Folin&Ciocalteu's phenol reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolicreducing substances to form chromogens that can be detected spectrophotometrically.The color development is due to the transfer of electrons at basic pHto reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lowervalence. The probable reaction is given here (only phenolic portion of Tyrosine is shown here and one probable oxidized structure is shown here).**Please note that Cysteine and Tryptophan also respond to this test and Cystine also shows mild bluepurplecolor.**



Lead Acetate test for Cysteine:

Sulphur containing amino acids, such as cysteine, upon boiling with sodium hydroxide (hot alkali) yield sodium sulphide. Then lead acetate solution is added and boiled for some time. Due to the conversion of the organic sulphur to inorganic sulphide, which can be detected by the black precipitation of lead sulphide, confirms sulphur containing amino acid.

Please note that Methionine does not respond to this test butCystine responds to this test.

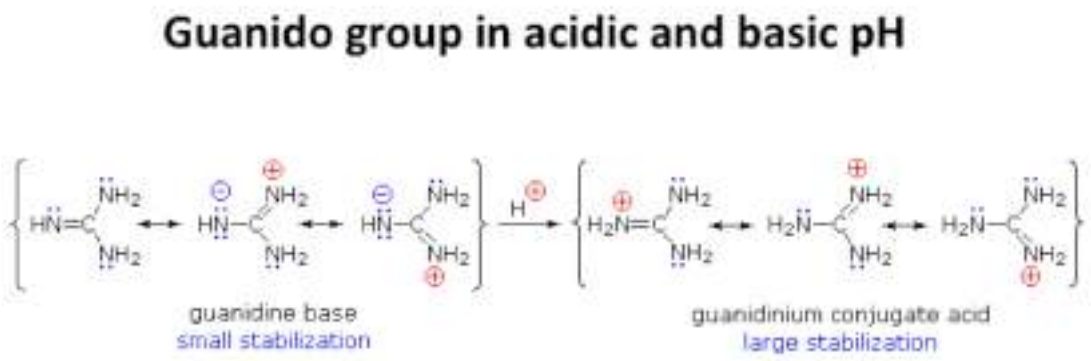
$$S.(Protein) + 2\ NaOH \rightarrow Na_2S$$

$$Na_2S + (CH_3COO)_2Pb \rightarrow PbS\downarrow (\textbf{Black}) + 2\ CH_3COONa$$

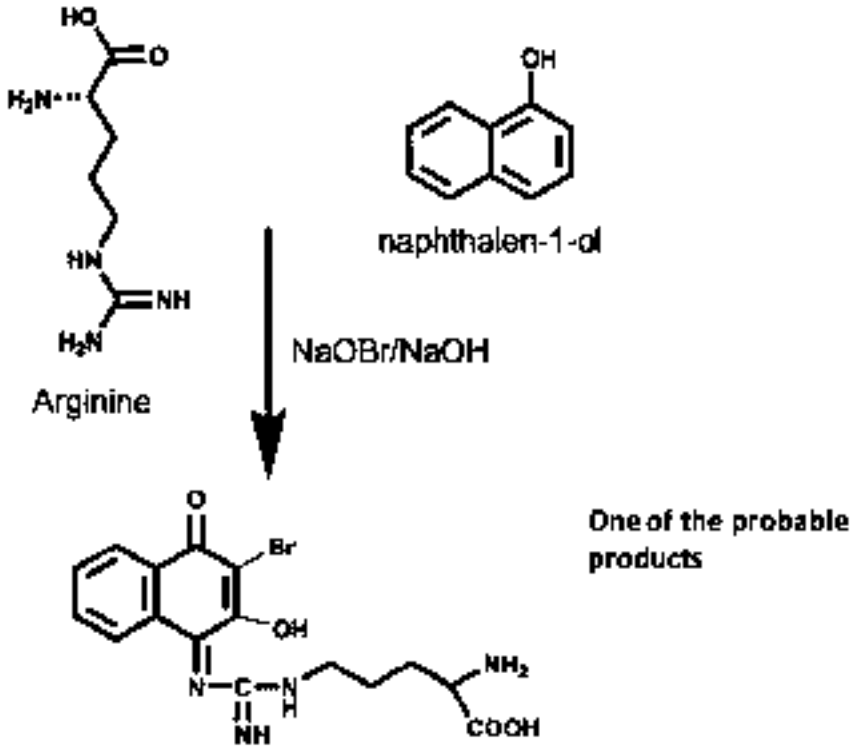
Scheme 1: Black PbS formation in $PbOAc_2$ test.

Sakaguchi test for Arginine:

The only amino acid, which contains a guanidine group, is arginine. Arginine gives a red color with α-naphthol, in the presence of an oxidizing agent like Bromine solution. This testis specific for the presence of guanidine. The NaOH helps to bring the arginine in Zwitterionic form and that undergoes a condensation reaction with α–napthol and develops **red**/**wine**color.



Possible mechanism



Spectral properties of amino acids:

Trp, Tyr, and Phe contain conjugated aromatic rings. Consequently, they absorb light in the ultraviolet range (UV). The extinction coefficients (or molar absorption coefficients) of these three amino acids are:

Aminoacid	Extinction Coefficient ε (at I_{max})
Trp	5,050 $M^{-1}cm^{-1}$ (280 nm)
Tyr	1,440 $M^{-1}cm^{-1}$ (274 nm)
Phe	220 $M^{-1}cm^{-1}$ (257 nm)

The amount of light absorbed by a solution of concentration [X] is given by the Beer-Lambert Law:

$$A = \log \frac{I_0}{I} = \varepsilon [X] l$$

Where,
A is termed the "absorbance" of the sample;
I_0 is the intensity of the incident light;
I is the intensity of the light that leaves the sample;
ε is the molar extinction coefficient at a specific wavelength, *e.g.* at I_{max};
[X] is the concentration of the absorbing species; and
l is the path length (usually 1 cm).

A solution that does not absorb any light ($I=I_o$) has an absorbance of 0. A solution that absorbs most of the light that passes through it, has a large absorbance. For example, if 90% of the light were absorbed, $I_o/I = 10$, and $A = 1.0$.

The above table shows that Trp absorbs UV light the strongest. Furthermore, since both Trp and Tyr show the maximum light absorbance at approximately 280 nm the absorption maximum of most proteins is around 280 nm. In contrast, the absorption maximum for nucleic acids is approximately 260 nm.

Experimental Procedure:

Use test tubes for each test. Sodium hydroxide and Bromine are specially corrosive and toxic. Please be very careful when handling these. When you are required to heat reaction

solutions, place the reaction solutions in boiling-water bath. Please do not touch the hot surface or knock the bowl over!

a) Erlisch's test for tryptophan:

To 1.0 mL of 1% amino acid solution, mix 1 mL of Erlisch's reagentand shake well. Few drops of conc. HCl are added and the mixture is heated in the waterbath for 7-8 minutes. Report color.

The *p*-dimethylaminobenzaldehyde reacts with tryptophan to form a red coloured product. Initially it is **red/ pink** and gradually it becomes **pinkish blue**.

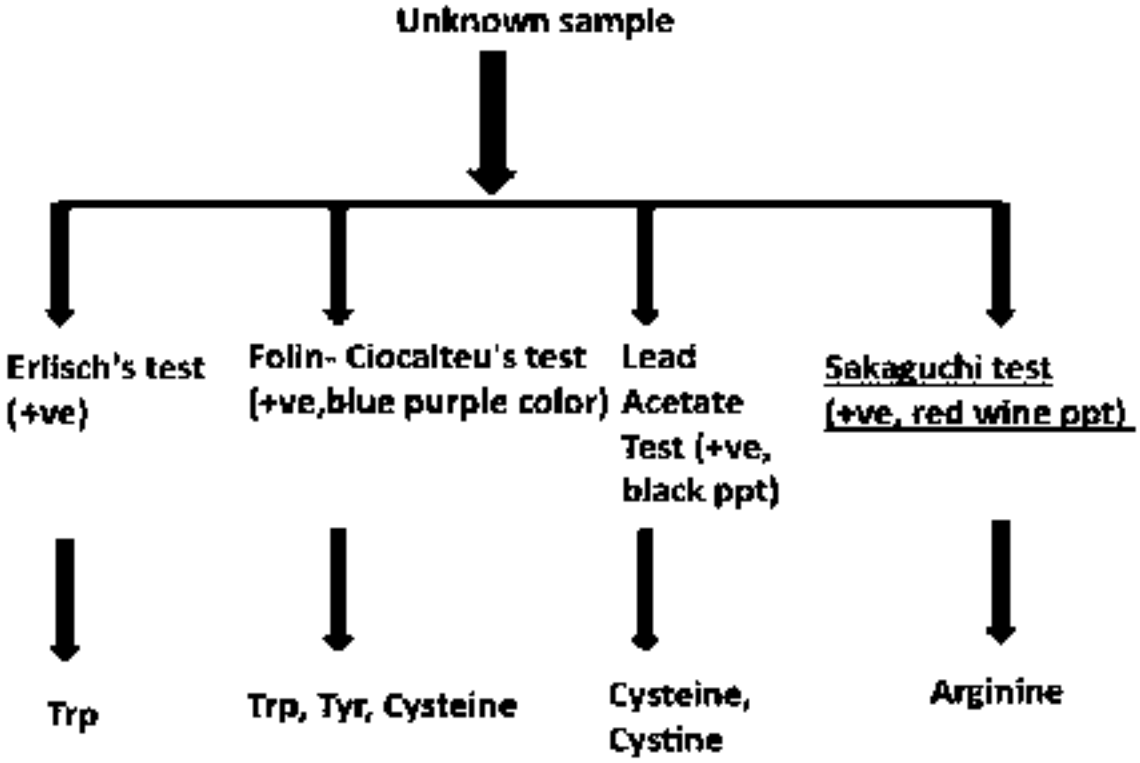
b) Folin-Ciocalteu's test for Tyrosine:
To 100 µl of 1% amino acid solution add 100 µl of Folin-Ciocalteu's reagent. Mix the contents well and add 500 µlof saturated sodium carbonate solution.The phenolic group of tyrosine residue (amino acid) in a protein will produce a **bluepurple**color complex.

Please note that Cysteine and Tryptophan also respond to this test and for Arginine,Cystine it gives faint blue colour.

c) Lead acetate test for Cysteine:
Mix 500 µl of 5% amino acid solutionwith 500 µl of 40% NaOH and 10 drops of 1% lead acetate solution. Boil the reaction mixture for 8 -10 mins in water bath and cool the contents under tap water.Due to the conversion of the organic sulphur to inorganic sulphide, which can be detected by the **black precipitation** of lead sulphide, confirms sulphur containing amino acid. See against the white paper then it will be clearly visible.

Please note that Methionine does not respond to this test butCystine responds to this test.

d) Sakaguchi test for Arginine:
This reaction should be performed inside the hood. To 500 µl of 1% amino acid solution cooled in ice bath mix, add 1.0 mL of 10% NaOH and 1.0 mL of 0.02% alpha-naphthol solution. **Add NaOH and alpha-naphthol solution in ice.** Shake well and add 8-10 drops of alkaline hydrobromide solution. Observe color. It develops **red**/**wine**color.


Unknown sample
Erlisch's test (+ve)
Folin- Ciocalteu's test (+ve,blue purple color)
Lead Acetate Test (+ve, black ppt)
Sakaguchi test (+ve, red wine ppt)
Trp
Trp, Tyr, Cysteine
Cysteine, Cystine
Arginine

Qualitative Analysis of Amino Acids

Unknown Sample	Erlisch's test	Folin-Ciocalteu's test	Lead acetate test	Sakaguchi test
Tryptophan	Pinkcolour gradually turned topurple blue Trp present.	Purple blue color W/Y/C may be present	No black ppt, No colour Cys absent	Brown color Arg absent. Trp may be present.
Tyrosine	Yellowish green Trp absent	Purple blue color W/Y/C may be present	No black ppt, No colour Cys absent	-ve Initially no color but graduallyitturned to Red winecolour Tyr may be present
Cysteine	Yellowish green Trp absent	Purple blue color W/Y/C may be present	Black ppt in suspension Cys present	No colour Arg absent. Cys may be present.
Arginine	Yellowish green Trp absent	Faint blue color Arg may be present	No black ppt, No colour Cys absent	Red winecolourappeared immediately. Arg present.

Aromatic compound and Huckel's Rule

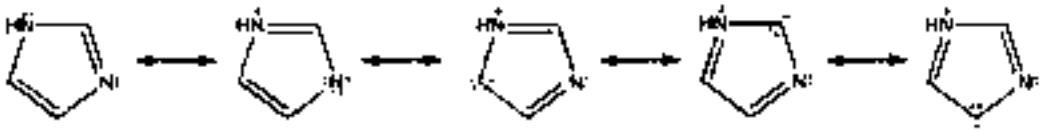
An aromatic (or aryl) compound contains a set of covalently bound atoms with specific characteristics:

A delocalized conjugated π system, most commonly an arrangement of alternating single and double bonds

Coplanar structure, with all the contributing atoms in the same plane

Contributing atoms arranged in one or more rings
A number of π delocalized electrons that is even, but not a multiple of 4. That is, 4n + 2 number of π electrons, where n=0, 1, 2, 3, and so on. This is known as Hückel's Rule.

Aromaticity ofHistidine



pKa concepts

The pKa is a measure of the strength of an acid. Specifically, it's the negative log of the dissociation constant for an acid in water.

The pKa of hydrochloric acid is around -7. Solutions of HCl at 1M, 0.1M, and 0.01M would have pHs of 0, 1 and 2 - respectively. However, in all three solutions the pKa of HCl is -7. The pH is a measure of acid in solution. The pKa is a measure of how strong an acid is.

For a weak acid, which dissociates as follows:

$$HA \leftrightarrow H^+ + A^-$$

$$\text{equilibrium constant} = K_{eq} = K_a = \frac{[H^+] \times [A^-]}{[HA]}$$

An interesting and extremely useful relationship between pH and pK_a can be obtained simply by taking logarithms (to the base 10) of the previous equation:

$$\log_{10}K_a = \log_{10}[H^+] + \log_{10}[A^-] - \log_{10}[HA]$$

Therefore

$$-\log_{10}[H^+] = -\log_{10}K_a + \log_{10}[A^-] - \log_{10}[HA]$$

Note: log a – log b = log (a/b)

giving the Henderson-Hasselbalch equation:

$$pH = pK_a + \log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

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The most convenient form of this Henderson-Hasselbalch equation, is

$$pH = pK_a + \log_{10}\left(\frac{[\text{conjugate base}]}{[\text{conjugate acid}]}\right) = pK_a + \log_{10}\left(\frac{[\text{proton acceptor}]}{[\text{proton donor}]}\right)$$

Using pKa and pH relationship

- By using pK_a values, we are able to express the strength of an acid (i.e. its tendency to dissociate) with reference to the pH scale.
- If K_a is large, then pK_a will have a *low* numerical value. E.g.,

  Hydrochloric acid, HCl has a $pK_a = -7$

  Acetic acid, CH_3COOH has a $pK_a = 4.77$
- A strong acid is one which is largely, or completely, dissociated, and which therefore has a *high* K_a value (and low pKa).
- A weak acid is one that is only slightly dissociated in solution, and has a low K_a value.

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if we consider the situation where the acid
is one-half (50%) dissociated,
or where
$[A^-] = [HA]$
(that is 50% negatively charged and 50% uncharged)
then, substituting in the
Henderson-Hasselbalch Equation

$$pH = pKa + \log(A^-/HA)$$

$$pH = pKa + \log(1)$$

Therefore $pH = pKa + 0$

and $pH = pKa$

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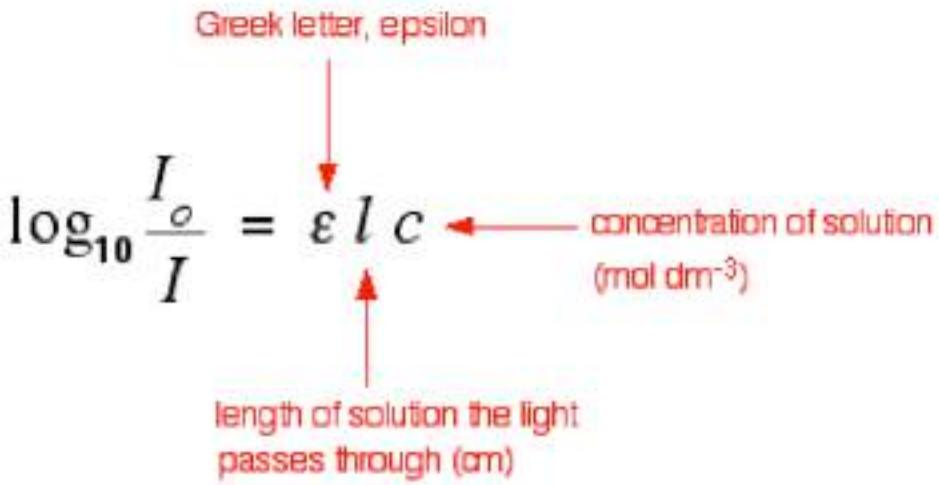
Greek letter, epsilon
$\log_{10} \frac{I_o}{I} = \varepsilon l c$
concentration of solution (mol dm^{-3})
length of solution the light passes through (cm)

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$$A = \varepsilon l c$$

$$A = \log_{10} \frac{I_o}{I} = \varepsilon l c$$

$$\varepsilon = \frac{A}{l c}$$ Molar absorptivity

the absorbance ranges from 0 to 1, but it can go higher than that.

An absorbance of 0 at some wavelength means that no light of that particular wavelength has been absorbed. The intensities of the sample and reference beam are both the same, so the ratio Io/I is 1. Log10 of 1 is zero.

An absorbance of 1 happens when 90% of the light at that wavelength has been absorbed - which means that the intensity is 10% of what it would otherwise be.

In that case, Io/I is 100/10 (=10) and log10 of 10 is 1.

The amount of radiation absorbed may be measured in a number of ways:

Transmittance, $T = P / P0$

% Transmittance, $\%T = 100\ T$

Absorbance,

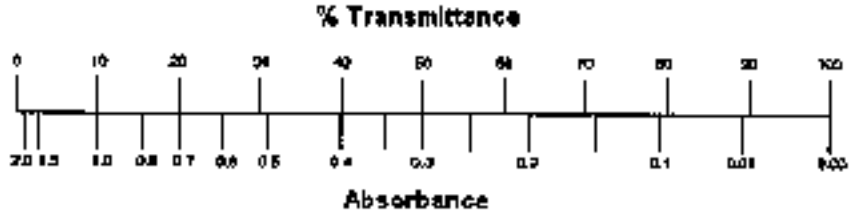
$A = \log10\ P0 / P$

$A = \log10\ 1 / T$

$A = \log10\ 100 / \%T$

$A = 2 - \log10\ \%T$

Calculate absorbance from the percentage of transmittance



So, if all the light passes through a solution without any absorption, then absorbance is zero, and percent transmittance is 100%. If all the light is absorbed, then percent transmittance is zero, and absorption is infinite.

Visible light consists of wavelengths ranging from 380 nm (blue violet) to 720 nm (red). When all wavelengths of visible light are present, the light appears "white" to our eyes. If any wavelength is removed (absorbed), we perceive the remaining combination of wavelengths of light as the "complimentary" color

Absorbed Wavelength (nm)	Absorbed Color	Perceived (Transmitted) Color
400 nm	violet green	yellow
450 nm	indigo	yellow
480	blue	orange
490	Blue-green	red
530	green	purple
570	Yellow-green	Dark blue
600	orange	blue
650	red	green

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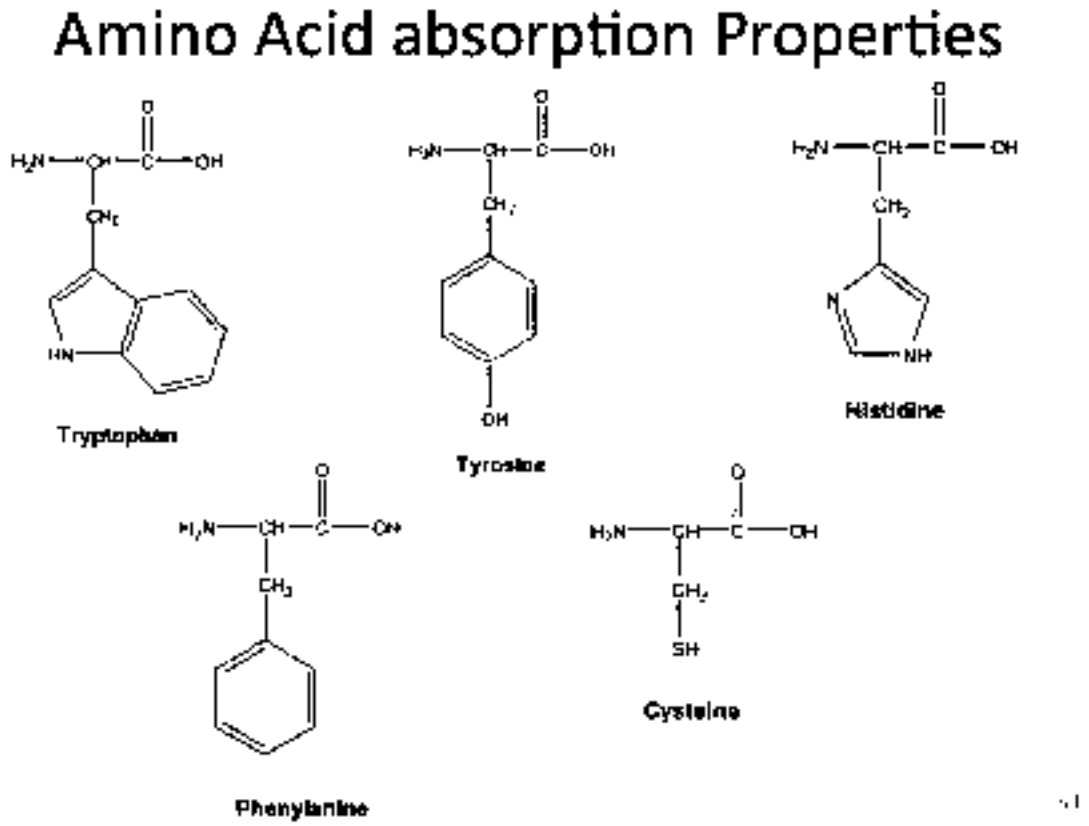
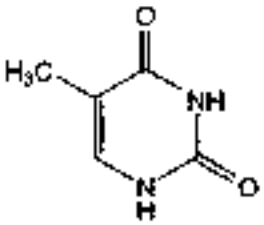


Peptide bond: 220 nm

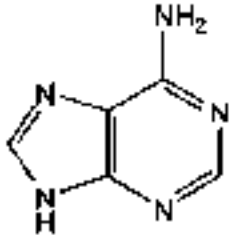
Trp: 280 nm ; Tyr : 270 nm; Phe : 254 nm

Common Absorbing Biochemicals

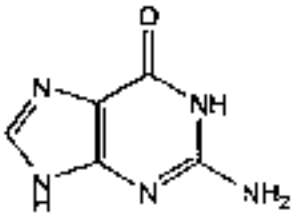
- The bases of nucleic acids



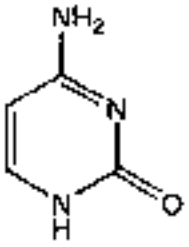
Thymine



Adenine



Guanine



Cytosine

Nucleic Acid Absorption Properties

Base	λmax (nm)	ε ($mM^{-1}cm^{-1}$)
Guanine	275	8.0
Adenine	260	12.9
Cytosine	265	5.8
Thymine	258	8.0

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Experimental Procedure (Qualitative Test of amino acids):

Use test tubes for each test. Sodium hydroxide and Bromine are specially corrosive and toxic. Please be very careful when handling these. When you are required to heat reaction solutions, place the reaction solutions in boiling-water bath. Please do not touch the hot surface or knock the bowl over!

a) Erlisch's test for Tryptophan:

To 1.0 mL of 1% amino acid solution, mix 1 mL of Erlisch's reagent and shake well. 5-6 drops of concentrated HCL are added and the mixture is heated in the water bath. Report color.

The *p*-dimethylaminobenzaldehyde reacts with tryptophan to form a red coloured product. Initially it is **pink** and gradually it becomes **blueish pink.**

b) Folin-Ciocalteu's test for Tyrosine:
To 100 µl of 1% amino acid solution add 100 µl of Folin-Ciocalteu's reagent. Mix the contents well and add 500 µl of saturated sodium carbonate solution. The phenolic group of tyrosine residue (amino acid) in a protein will produce a **blue purple** color complex.

Please note that Cysteine and Tryptophan also respond to this test and for Arginine, Cystine it gives faint blue colour (See against the white paper then it will be clearly visible).

c) Lead acetate test for Cysteine:
Mix 500 µl of amino acid (5% for cysteine and for others 1%) solution with 500 µl of 40% NaOH and 10 drops of 1% lead acetate solution. Boil the reaction mixture for 8 -10 mins in water bath and cool the contents under tap water. Due to the conversion of the organic sulphur to inorganic sulphide, which can be detected by the **black precipitation** of lead sulphide, confirms sulphur containing amino acid. **See against the white paper then it will be clearly visible.**

Please note that Methionine does not respond to this test but Cystine responds to this test.

d) Sakaguchi test for Arginine:

Note: First place the tubes in ice bucket then add Amino Acids and all the reagents as mentioned below. Do not take out the tube from Ice.
To 500 µl of 1% amino acid solution, add 1.0 mL of 10% NaOH and 1.0 mL of 0.02% alpha-naphthol solution. Shake well and add 8-10 drops of alkaline hydrobromide solution. Observe color. It develops **red**/**wine** color.

N.B.: Observe the result immediately

Results:

Unknown Sample	Erlisch's test	Folin-Ciocalteu's test	Lead acetate test	Sakaguchi test
Tryptophan	Pink colour gradually turned to blueish pink Trp present.	Purple blue color W/Y/C may be present	No black ppt, No colour Cys absent	Brown color Arg absent. Trp may be present.
Tyrosine	Yellowish green Trp absent	Purple blue color W/Y/C may be present	No black ppt, No colour Cys absent	Initially no color but gradually it turned to Red/Wine colour Tyr may be present
Cysteine	Yellowish green Trp absent	Purple blue color W/Y/C may be present	Black ppt in suspension Cys present	No colour Arg absent. Cys may be present.
Arginine	Yellowish green Trp absent	Faint blue color Arg may be present	No black ppt, No colour Cys absent	Red/Wine colour appeared immediately. Arg present.