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Pigment Identification during deetiolation of barley seedlings

Abstract

Angiosperm plants like barley(*Hordeum vulgare*) lack chlorophyll when grown in complete dark. Upon exposure to light, chlorophyll is formed during the process of deetiolation. Here , we show that in complete darkness barley seedlings accumulate monovinyl protochlorophyllide. Also small amounts of a second protochlorophyllide form were found. Spectroscopic measurements indicated that the unknown pchlide form is divinyl protochlorophyllide. Upon exposure to light for ten seconds some of MV-protochlorophyllide was photoreduced to MV chlorophyllide while divinyl pchlide remained constant. The loss of chlide and accumulation of chlorophyllide a was determined. Within twenty minutes MV-pchlide concentration was restored , the concentration of chlide was strongly decreased and chl continued to accumulate. Interestingly, exposure of plants to safelight was not capable to phototransform MV-Pchlide. However it resulted in increase in the second protochlide form upon exposure of barley seedlings to light. The finding indicates that the safelight used in the experiment influences capacity of chlorophyll biosynthesis.

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1.**Introduction:**

Barley plants have yellow leaves when they are grown in dark due to the absence of chlorophyll. Detiolation is defined in Wikipedia as "De-etiolation, on the other hand, is a series of physiological and biochemical changes a plant shoot undergoes in response to sunlight". It simply means the process by which the plants kept in dark become green when exposed to light. Angiosperms like *Hordeum vulgare* L do not form chlorophyll if grown only in darkness. The main component seen in the dark grown plants (here barley) is the presence of protochlorophyllide which is converted to chlorophyllide when plants are exposed to light. However, there can be both light dependent reduction of protochlorophyllide and light independent reduction of protochlorophyllide. The light dependent reduction of protochlorophyllide is seen especially in the flowering plants. The light independent reduction of protochlorophyllide is found specially in non flowering lower plants, green algae and bacteria. The enzyme LPOR (Light depentdent Protochlorophyllide Oxidoreductase) is required for the reduction of protochlorophyllide in etioplast in light dependent mostly angiosperm plants and anoxygenic photosynthetic bacteria and also in algae, mosses, ferns and gymnosperms which reduce protochlorophyllide in dark have enzyme called DPOR (Dark operated Protochlorophyllide oxidoreductase) (Willows 2003).

It is very interesting to know the genetic components that play the roles in the process of greening. If we examine the greening of flowering plants in the genetic level, *por* gene which codes Light-dependent oxidoreductase is responsible for greening of the plants(Willows 2003). It is seen that there are mainly three genes of *por* called the *por A , por B* and *por C* which code their respective POR A , B and C enzymes. The *por A* gene is expressed in darkness and the enzyme POR is formed mostly in dark grown plants (Rudiger 2003). Enzyme LPORA which is the isoform of enzyme LPOR(Light dependent Protochlorophyllide Oxidoreducatse) specially found in barley and *A.thaliana* plays a important role in the deetiolation of the seedlings. The *por B gene* is expressed in the dark and in the light. But this gene is not found in barley (Rudiger 2003). The *por C* is only expressed in light. In complete dark, the components like LPOR, NADPH, and protochlorophyllide forms a ternary complex and when light falls in the plant, protochlorophyllide is reduced to chlorophyllide.(Willows 2003) This ternary complex is membrane associated and forms a crystalline like structure called the prolamellae bodies (PLBs) which is found in the etiolasts(Willows 2003). Due to this structure, the spectral form of protochlorophyllide is seen. (Willows 2003) so it can be said that the formation of PLB is directly related to the formation of protochlorophyllide by the plants. It has been found that the lipids are also essential for the formation of PLBs (Willows 2003). When the light falls the protochlorophyllide is converted to chlorophyllide and then to chlorophyll. This change is also observed in the morphological changes in the

prolamellae bodies which are dispersed and disaggregated.(Willows 2003) In the both process which involves the formation and the process of disaggregation and dispersal is associated with the protein phosphorylation (Willows 2003). It was found that the inner membranes of etioplasts called prolamellar bodies contained mostly POR proteins before illumination and the thylakoids after illumination. This suggests that the POR protein moves from prolamellar bodies to prothylakoid after illumination with the light that leads to the formation of chlorophyllide from protochlorophyllide. The pathway of synthesis of chlorophyll is completed with the esterification of chlorophyllide with phytol (Willows 2003). The esterification can take place either with phytyl pyrophosphate or geranylgeranyl pyrophosphate in the presence of chlorophyll synthase (Willows 2003). The reduction of geranylgeranyl to form phytyl can take place either before or after the esterification of chlorophyllide(Willows 2003).

In the study of different pigments it should be considered that protochlorophyll is one of the degraded pigment but not the precursor of chlorophyll. The structure of it is understood as phytyl ester of Mg-vinylpheoporphyrin (Rudiger 2003)

The biosynthesis of chlorophyll can be characterized when the dark grown plants are illuminated with strong flash of light and dark incubated for different period of time. It is reported that the photo conversion by Light dependent Protochlorophyllide oxidoreducatase in the presence of light takes place by single photon of light in a single photochemical reaction (Willows 2003). But all the protochlorophyllide are not converted to chlorophyllide by a single flash of light(Willows 2003). Based on ability to be converted to chlorophyllide, two forms of protochlorophyllide have been suggested : one which has a fluorescence emission maxima range between 638-645 nm which may be due to the formation of dimer and the second one which has a emission maxima range between 650-657 nm which is postulated as aggregated form and is said to be dependent on the lipid binding.(Willows 2003).

In this experiment , different pigments were extracted from 4.5 days old barley plants which were grown in dark and subsequently exposed for ten seconds and dark incubated to different time period upto sixty minutes using DMF (N,N-Dimethyl formamide). The pigments are characterized based on its mobility in the hydrophobic reverse phase chromatography, absorbance maxima in spectrophotometer and the excitation and emission spectrum of fluorescence spectrophotometry. The main motive of this experiment is to observe the changes that occur when a dark grown barley plant is cut and illuminated to flash of light and each sample is incubated for different time interval at dark. It has been already found in several literature that dark incubation plays a crucial role in the photoconversion of protochlorophyllide to chlorophyllide. So the results of this experiment could also be further analysed based on the different pigments observed at different time interval of dark incubation. Based on the different

properties of pigments the biosynthetic pathway of chlorophyll is explained and compared with the result.

2.Theory:

2.1.1 Pigments:

2.1.1.1Chlorophyll

Chlorophyll is the the most abundant green pigment on Earth. An average plant leaf has around 70 million cells with 5 billion chloroplasts, each containing about 600 million molecules of chlorophyll. Chlorophyll traps the energy due to the presence of central molecule which converts the radiant energy to chemical energy**.** Chlorophyll is green pigment found in almost all plants, algae and cyanobacteria. It obtained its name from greek words “chloros” meaning green and “phyllon” meaning plant. Chlorophyll molecules are arranged in and around photosystems that are embedded in the thylakoid membrane of chloroplasts. (simpson and knötzel, 1996)

The main function of chlorophyll is to obtain energy from light. Chlorophyll absorbs light most strongly at the blue portion of electromagnetic spectrum. However it is poor absorber of green or near green portion of light. Hence the green color of chlorophyll-containing tissues. It helps to transfer the light energy by resonance energy transfer to specific chlorophyll pair in the reaction center of the photosystem.

Chlorophylls are composed of a chlorin moiety and a long alcoholic carbon chain which is usually diterpene phytol. All chlorophyll have a magnesium ion in the centre of tetrapyrrole ring. They also have propionate group at c17 and a esterified phytol.

Chlorophyll a has vinyl group on $C3^{b}$, methyl group on C7, ethyl group C8 , methyl group on C12 , methyl easter on $C13^{2}$, propionyl phytol on $C17^{d}$.Chlorophyll *b* has formyl group in C7 position instead of methyl group in chlorophyll *a.*

Chlorophyll a is present in light harvesting antenna pigment complexes and in the reaction centre complexes . it functions as an accessory pigment as well as the primary electron donor in the reaction centres of photosystems I and photosystems II . The chlorophyll a pigment without magnesium ion is called pheophytin and without both magnesium ion and phytol is called pheophorbide.(Fujita 2002)

Phytol is a long hydrocarbon (C20). It is formed from an isoprenoid which is formed from four units of isoprene. The presence of phytol group gives the hydrophobic characteristic to the chlorophyll pigments.(Fujita 2002).

Chlorophyll b is present in the light-harvesting antenna complexes , mainly in the major antenna of PSII and is absent in the reaction centre. All plants and green algae have 25% of their chlorophyll as chlorophyll b (Willows 2003). Chlorophyll b has a formyl group in C7 of tetrapyrole ring instead of methyl group.

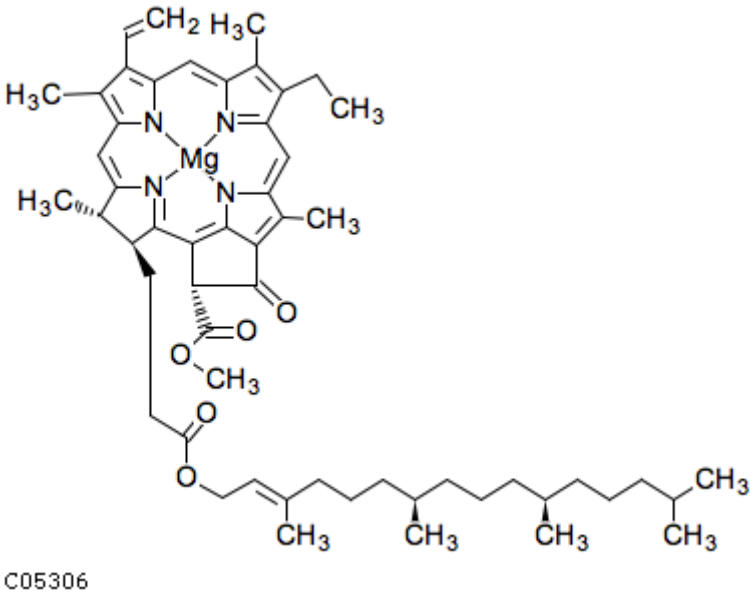

Figure 1: Chlorophyll molecule

(source: http://www.kegg.jp/dbget-bin/www_bget?C05306)

2.1.1.2 Protochlorophyllide

Protochlorophyllide is the precursor molecule of the chlorophyll which lacks the phytyl side chain in the C17 carbon .It has a double bond between C17 and C18 in the twenty membered tetrapyrrole structure. It is flurorescent. Based on ability to be converted to chlorophyllide, two forms of protochlorophyllide have been suggested : one which has a fluorescence emission maxima range between 638-645 nm which may be due to the formation of dimer and the second one which has a emission maxima range between 650-657 nm which is postulated as aggregated form and is said to be dependent on the lipid binding.(Willows 2003)

The conversion of protochlorophyllide to chlorophyllide a is done by protochlorophyllide reductase. Subsequently it is converted to chlorophyll a. The conversion of protochlorophyllide to chlorophyll is a light dependent reaction. It can occur in dark as well if it has a light independent protochlorophyllide reductase. It is also called Dark operative protochlorophyllide oxidoreductase (DPOR). Angiosperms have lost DPOR. (Willows 2003)

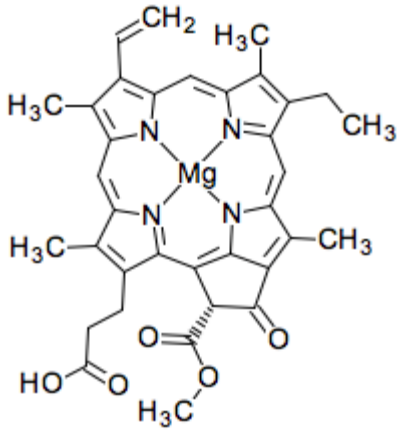

Figure 2: Protochlorophyllide

Source:http://www.kegg.jp/dbget-bin/www_bget?C02880

2.1.1.3 Chlorophyllide a:

The chlorolophyll a lacking phytol group is chlorophyllide a .

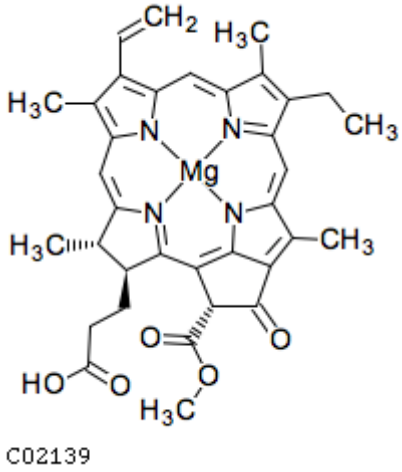

Figure 3 : Chlorophyllide a.

(source http://www.kegg.jp/dbget-bin/www_bget?C02139)

Presence of propanoic acid at the top position of c17 of the molecule.

C17 and C18 is reduced .there is no double bond

2.1.1.4 Structure of pheophytin

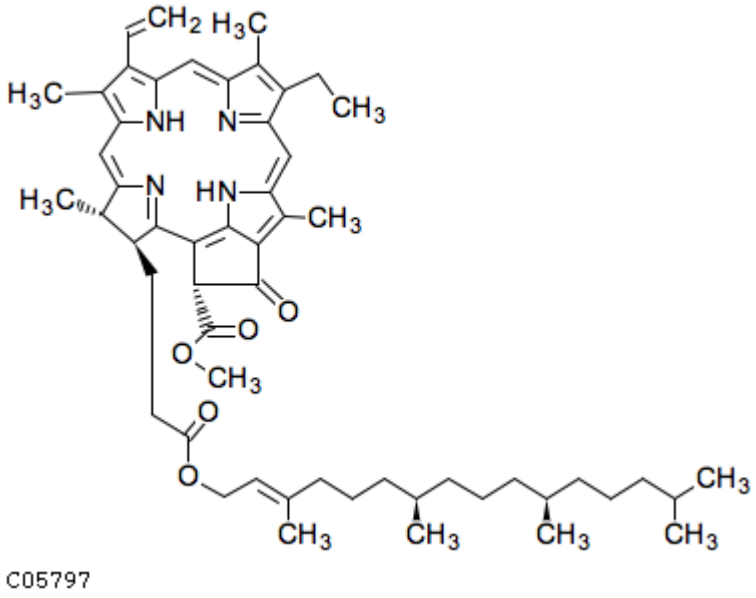

Figure 4: structure of Pheophytin a

(source http://www.kegg.jp/dbget-bin/www_bget?C05797)

Pheophytin a is derivative of chlorophyll a lacking the magnesium ion.

2.1.2 Chlorophyll biosynthetic pathway:

It is reported that chlorophyll a of green plants is formed via four parallel biosynthetic pathway. It has been called fully esterified and acidic branches. It is further divided into monovinyl and divinyl sub groups(Belanger, Duggan et al. 1982).

Tetrapyrrole Biosynthetic pathway:

Chlorophyll, bacteriochlorophyll and their derivatives formed as variants of the evolutionary conserved biosynthetic pathway of tetrapyrroles which consists of at least

18 different enzymatic steps. The first committed precursor of chlorophyll is 5-Amino laevulinic acid (ALA) (Grimm). It is synthesized from glutamate in most bacteria and plants. There are two different pathways for ALA formation. The one way is the one in which ALA synthase acts as a catalyst . this pathway is found in α proteobacteria and eukaryotic organisms that lack chloroplast (Fujita 2002). The other pathway is called the C_5 pathway in which three enzymes are used to covert glutamic acid to ALA. The two molecules of ALA are condensed to form one molecule to porphobilinogen. The four molecules of porphobilinogen are fused together to form hydroxymethylbilane.(Grimm) The hydroxymethylbilane is converted to uroporphyrinogen III. The uroporphyrinogen III is oxidized to coproporphytinogen III by the action of enzyme uroporphyrinogen III decarboxylase. Coproporphyrinogen III oxidase converts two propionic acid side chains into vinyl groups to form protoporphyrinogen IX by protoporphyrinogen IX oxidase. Protoporphyrin IX is directed to the chlorophyll or the haem-synthesizing pathway. For chlorophyll formation, Mg-chelatase inserts Mg2+ into protoporphyrin IX called the Mg-protoporphyrin IX by a complex ATP-dependent catalytic reaction. Magnesium chelatase is a three subunit enzyme. Magnesium protoporphyrin IX is converted to Mg-protoporphyrin IX monomethyl ester by methylation at the propionic acid side chain on ring C .(Fujita 2002). 13-methyl propionic acid side chain undergoes a complex reaction to form fifth ring of chlorophyll. The reaction is proposed to be undergone in three steps:1.at C13' position there is formation of hydroxyl group 2. The hydroxyl group is converted to keto group by oxidation 3. The ring closure reaction leads to the formation of the fifth ring of the chlorophyll.(Fujita 2002) Thus the final molecule formed is 3,8-divinyl protochlorophyllide. Chlorophylls have a single vinyl group at C3 position and ethyl group at c8. Divinyl protochlorophyllide is reduced at the C8 vinyl group to yield monovinyl protochlorophyllide by vinyl reductase.(Grimm)

In angiosperms, the double bond between C17 and C18 in ring D of the macrocycle is reduced in a light dependent manner to form chlorophyllide a by protochlorophyllide oxidoreductase.(Grimm) In other photosynthetic organisms, protochlorophyllide are reduced by light independent. The chlorophyll synthesis which involves the intermediate geranylgeranyl diphosphate for the synthesis of phytyl diphosphate by geranylgernyl reductase of the isoprenoid pathway . Chlorophyllide a is esterified with a phytol or granylgenaniol chain which is catalysed by chlorophyll synthase.

Chlorophyll b is synthesized by oxidation of the methyl group of chlorophyll a to a formyl group via a hydoxymethyl chlorophyllide a. Chlorophyllide a oxygenase acts as a catalysis which depends on the NADPH and oxygen (Rudiger 2003) .

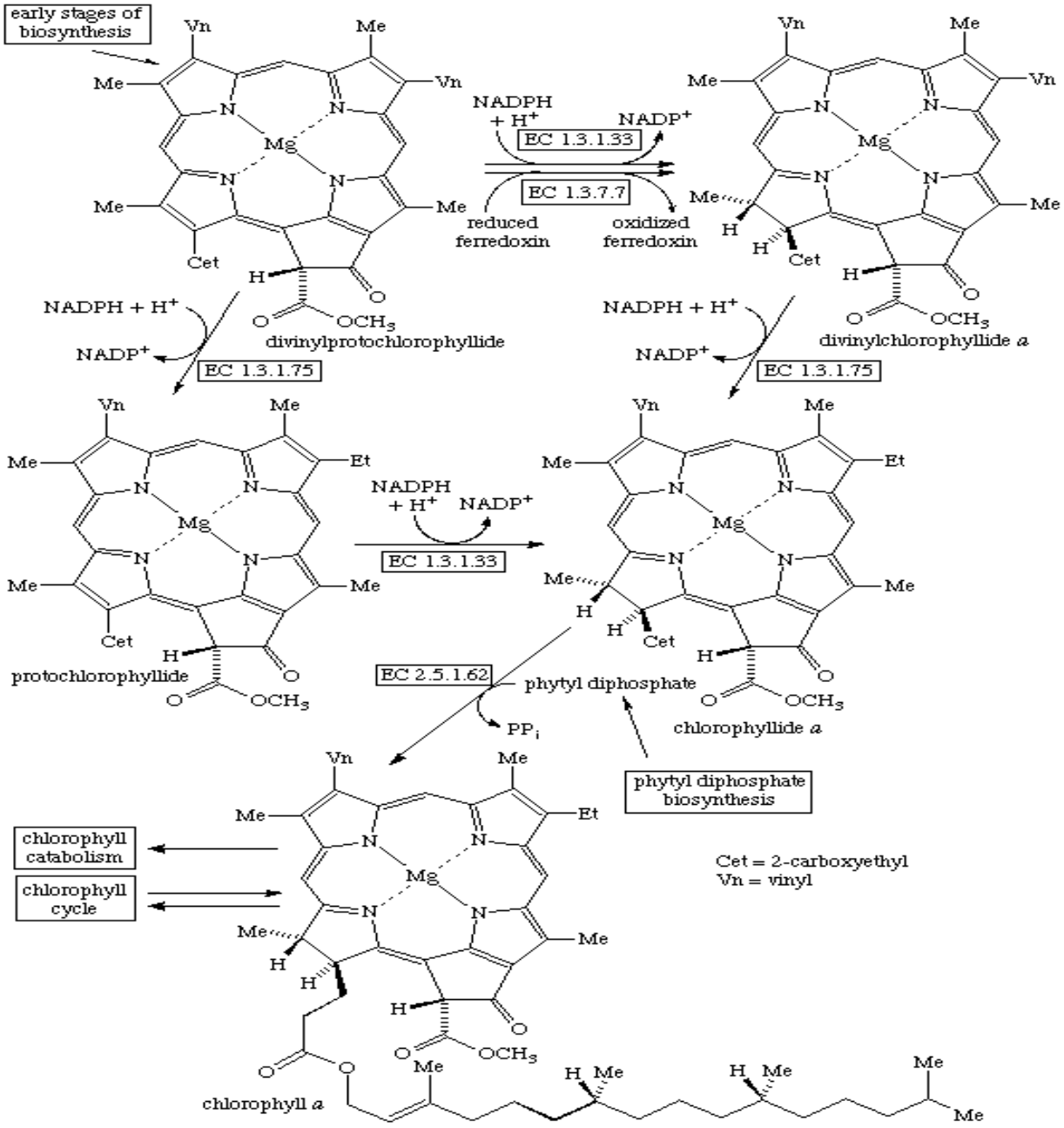

Figure 5 : Chlorophyll biosynthetic pathway

Source (http://www.kegg.jp)

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Geranylgeraniol is a diterpene alcohol .

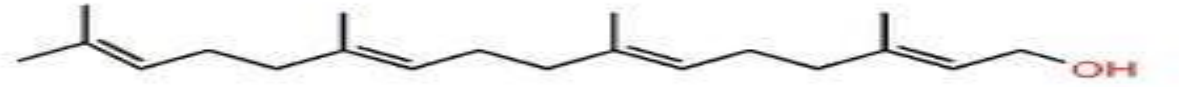

Figure 6:Geranylgeraniol molecule (www.wikipedia.org)

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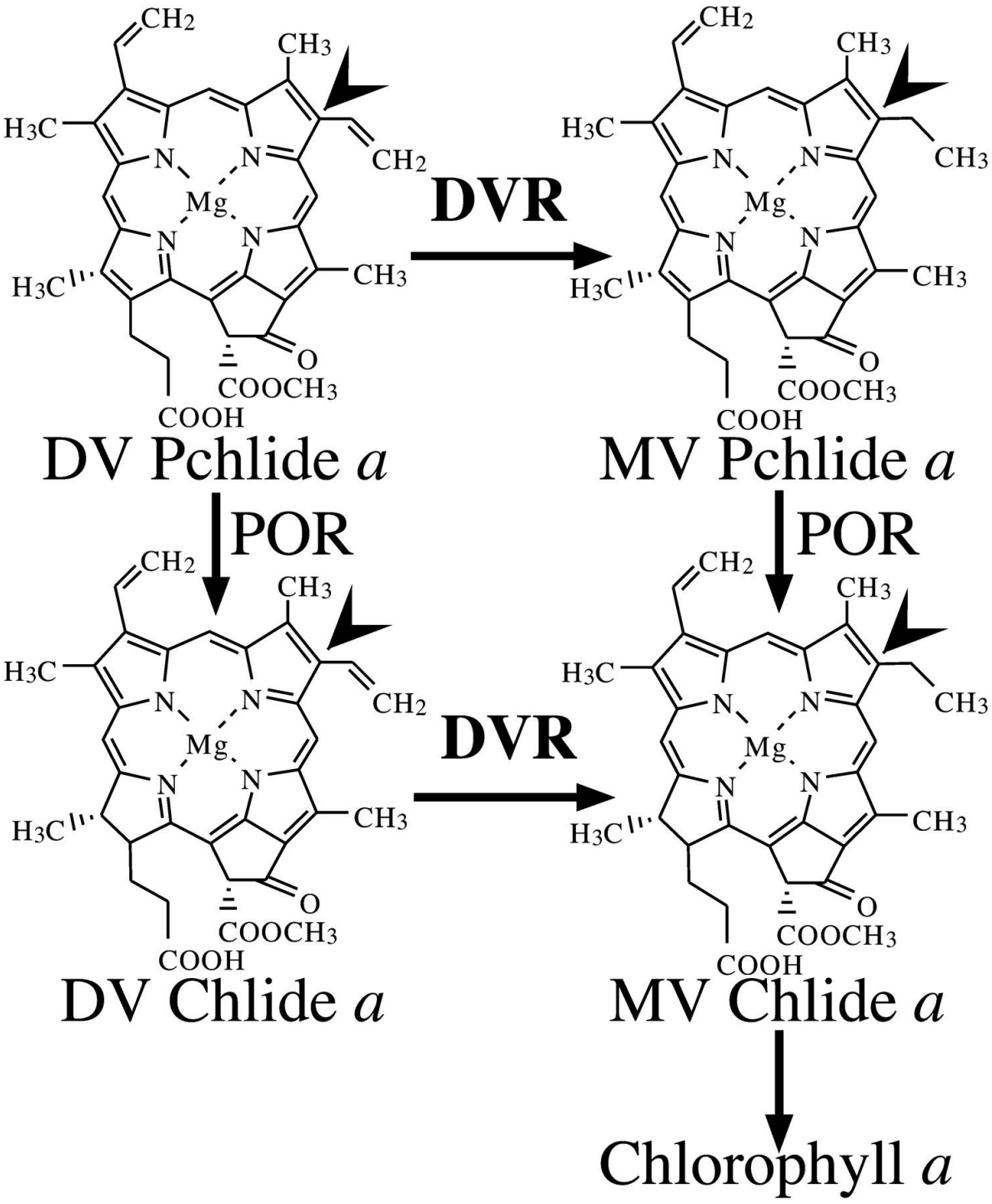


Figure 7: Chlorophyll synthesis pathway

Source (http://www.kegg.jp)

Shibata shift:

The shift in maxima absorbance wavelength from 684 nm to 672 nm is called the shibata shift. The maxima absorbance at 684 is due to the complex of POR, $NADP^+$ and chlorophyllide. Free Chlrophyllide a has an absorbance maxima at 672 nm. Shibata shift can be avoided in plant by addition of fluoride. This indicates that Phosphatase reaction is involved in this process which is often explained as the disaggregation. The shift can be made to occur in intact etioplast when phytyl diphosphate is allowed to be esterified to chlorophyllide a (Rudiger 2003).

2.2 Methods:

2.2.1 Pigment Extraction:

A.chlorophyll and carotenoid pigment:

Chlorophyll and carotenoid molecules are usually rather hydrophobic compounds so they can be extracted with a single or a mixture of organic solvents using homogenizer(Schoefs 2004). The chlorophyll, chlorophyllide, protochlorophyllide , protochlorophyll pheophorbide were extracted by using DMF(N,N-Dimethylformamide).

Extraction of chlorophyll by N,N DMF

The solvent is especially efficient when pigment concentration is low; time and tools are saved and the loss of pigment that usually occurs in more complicated extraction procedures is prevented. Grinding and centrifugation of tissues require a relatively high minimium volume of solvent which in effect lowers the concentration of pigment in the final solution (Moran and Porath 1980). DMF, an organic solvent in which Chl is soluble (Seely and Jensen 1965), was used to extract Chl from an alga (Volk and Bishop 1968) and from leaves of higher plants without the benefit of grinding(Back and Richmond 1969). Extraction of photosynthetic pigments with DMF is simpler than with acetone and the results are comparable(Moran and Porath 1980). All operations were carried out under green safelight. The extracts were stored in dark for 24-48 hour at 4 ˚C prior to spectroscopic examination. Both Pchl and chl extracts in DMF remained quite stable during storage. (Moran and Porath 1980)

Protochlorophilide extraction using DMF:

Both DMF and 80% acetone extracts were prepared for Pchl determination by direct immersion of the cotyledons in the solvents. The maxima of pchl at the red band in the DMF extracts is located at 626.0nm. (Moran and Porath 1980).

Chlorophyll a extraction using DMF:

The extract for chlorophyll a were prepared by direct immersion of the cotyledons in the solvents which had been exposed to ten minutes white light prior to removing their cotyledons The position of the maxima of pure Chl a in DMF is 664.6 and in the blue region is 433 nm. .(Moran and Porath 1980).

Chlorophyll b extraction using DMF:

Chlorophyll b is not present in detectable amounts in etiolated seedlings within the first hours following the light exposure.(SUNDQVIST 1974)

DMF is more efficient in extracting Pchl and chl a from intact etiolated cotyledons than acetone. Even when both extracts were prepared by first grinding the plant material, higher pigment yield was obtained with DMF than with acetone . No differences in pigment concentration were noted in DMF extracts prepared by grinding compared to those prepared by direct Immersion. (Moran and Porath 1980).

Angiosperm seedlings lack chlorophyll completely when grown in absolute darkness but become green upon exposure to light. The greening process implies the phototransformation of dark accumulated protochlorophyllide (Pchlide) to chlorophyllide (Chlide) catalysed by NADPH:protochlorophyllide oxidoreductase (POR) and the subsequent light-independent synthesis phytol-containing chlorophyll (Chl-phy, commonly called Chl). Soll et al. 1983; rudiger 1987

Phytalation is a precondition for the formation and accumulation of stable chlorophyll-protein complexes.Chl-Phy formation from chlide and geranylgeranyl diphosphate (GGPP) requires two enzymatic reactions, catalyzed by chl synthase and geranylgeranyl reductase, the products of the genes *ChlG* and *ChlP,* respectively.The recombinant Chl synthase accepts the substrates GGPP, producing Chl-GG, and phytyl diphosphate (PhyPP), producing Chl-Phy. Chl synthase of etiolated plants has preference for GGPP (Domanskii, Rassadina et al. 2003) and chl synthase of green plants a preference for PhyPP(Soll et al. 1983; rudiger 1987) leading to the assumption of a different order of prenylation and reduction in etiolated and green plants.

2.2.2 Chromatographic separation of pigments

Pigments contains chromophore. Chromophore is the part of the molecule which is involved in light absorption. It is made of several conjugated double bonds. Pigments

should be taken care during the extraction because they are highly sensitive to acid, base, oxygen, heat and light.

Chromatography is used to separate mixtures of molecules or ions. In the chromatography technique there are two phases, a moving substance called the mobile phase and a stationary substance called the stationary phase. The mobile phase flows past a stationary substance. The mixture containing the molecules to be separated is applied to one of the two phases.

2.2.2.1 Thin layer chromatography

Thin layer chromatography is considered as fast, effective and cheap method of pigment analysis. In a normal phase chromatography, the stationary phase is hydrophilic and has strong affinity for the hydrophilic molecules in the mobile phase. The hydrophilic molecules in the mobile phase has strong affinity with the stationary phase and the hydrophobic molecules are elulated first.

Migration rate:

As the molecules in the mixture move along at different speeds, they are gradually separated. The migration rate of a molecule is constant when using the same mobile – stationary phases, the identity of a particular molecule can be determined from the migration rate. (Domanskii, Rassadina et al. 2003)

2.2.2.2 High Performance Liquid Chromatography

Photosynthetic pigments, chlorophylls and carotenoids have a hydrophobic character. So, they are analyzed by C18 reverse phase columns.

2.2.2.3 Reverse phase chromatography

An elution procedure used in liquid chromatography in which the mobile phase is significantly more polar then the stationary phase,e.g. a microporous silica-based material with chemically bonded alkyl chains. The alkyl chains present in the stationary phase creates a significant hydrophobic stationary phase. Thus stationary phase has more affinity to the hydrophobic compounds. The hydrophobic molecule in the mobile polar phase adsorbs to the hydrophobic stationary phase and the hydrophilic molecule that is more strongly attracted to the mobile polar phase will pass through the column and is eluted first. The other molecules that are most strongly attracted to the immobile phase will move along slowest. As the molecules in the mixture move along at different speeds, they are gradually separated.Since the hydrophobic stationary phase is reverse of the normal phase chromatography, it is called the reverse phase chromatography. The mobile phase used in our experiment is methanol, acetone, and HEPES buffer.

The mobile phase used for the separation of hydrophobic molecules is usually made up of organic solvents .when polar molecules are present along with the mixture, a polar organic solvent mixed with a small amount of water is recommended.(In the experiment that I used for the separation of pigment, this later technique is used.)

Stationary phases:

Any inert non-polar substance can be used for the stationary phase. Commonly used stationary phase is octadecyl carbon chain also called C18 bonded silica and C8 bonded silica, cyano bonded silica, pure silica and phenyl bonded silica are also used.

Mobile phases:

Mixtures of water, aqueous buffer and organic solvents are commonly used to elute the reverse phase columns. The solvents must be miscible to water. The commonly used mobile phases are methanol, acetonitrile, tetrahydrofuran.

Sometimes heating of the column is used for improving the pigment separation. This is not recommended to separate chlorophylls and carotenoids, because heating can tigger carotenoid isomerization, chlorophyll epimerization and allomerization as well.

So the proper chromatographic conditions should be employed to ensure that the pigments or degradation products donot escape the analyses.

2.2.3 Detection of pigments:

The pigments that have been separated must be identified and quantified. The pigment identification is based on chromatographic analysis and spectral properties. The quantification is found by absorbance measurements.

The following are the mostly used techniques to identify and quantify the pigments:

2.2.3.1 UV-Vis spectroscopy:

The absorbance spectra of pigments can be considered as its fingerprint.

The environment of the pigment which includes solvent, temperature, ligation to protein, could influence the absorbance maximum and the shape of the spectrum. So for the accurate measurement of pigment concentration, the pigments should be dissolved into a solvent for which specific (or molar) absorbance coefficients have been determined.

The major limitation in the identification of pigments on the basis of absorbance is the overlapping of the absorbance bands of individual pigments.

Chlorophyll a absorbs light at wavelength range of 550-700nm and also belolw 480nm. By comparing with the specific absorption coefficient determined by MacKinney (Mackinney 1941), specific absorption coefficient of chl a in DMF at 664.5 nm was calculated to be 83.89 $g^{-1} l^{-1}$ (Moran and Porath 1980)

2.3.2 Fluorescence spectroscopy:

When the light falls in a chlorophyll, there is absorbance of energy of a photon depending on the wavelength. Due to the absorbance there is the energy transition from ground state to an excited state. This leads to the increase in the energy level of chlorophyll. The trend of transition is known by the absorption spectrum of the chlorophyll. If only one electron is allowed to stay in the higher energy orbital , this type of excitation state is called singlet. Because of the presence of two main excitation states called the first and second singlet states ,the chlorophylls like chlorophyll a and chlorophyll b have two main absorption maxima . At first the electron is elevated to first singlet state by the red light which has lower energy and bigger wavelength. The electron is elevated to the higher energy second singlet state by the blue light with much higher energy and smaller wavelength. After certain time, the electron from the second singlet state moves to the first singlet state and loses energy in the form of heat. The return of the electron from the first singlet state to the ground state takes place with the release of energy equal to the excitation energy. This is called fluorescence. It is seen that the fluorescence light has less energy than excitation light because some of the energy is released as heat energy. This is the basic principle of fluorescence.(Fujita 2002)

It can be concluded that spectroscopic methods can be used for primary identification but the identification of specific composition is not possible.

For the further identification , techniques such as chromatography can be used. Mass spectrometry can be used to characterize the structure of the molecule.

3.Materials and Methods

3.1 Materials Required

1. Cryoviles(1-14 numbered): A 3ml cryovile was used for the experiment. The cryoviles were numbered in the lid (to observe from top) and on the vile was covered by aluminium paper on all its side and numbered from outside.

2. Liquid Nitrogen Enclosed Box –A plastic box with a lid was used. The liquid nitrogen was poured in the box. The etiolated plants were cut and kept in a paper stand which was dipped in the box containing the liquid nitrogen. So it was named as liquid nitrogen box.

3. Dark box: A plastic box with a lid .It was used to keep the cryoviles containing the plant material after being exposed to the light.

4. Homoginizer: A glass rod that fits exactly in the 3ml cryovile to crush the leaves.

5. cutter(scapel)

6. Cutting Board: A glass cutting board with graduated lining.

7. light Chamber

8. Alarm Clock

9. Pippette (1ml):

10. Refrigerator(4 degree centigrade)

11. Marker

12. Tips :

13 Goggles

14. Aluminium foil

3.2 Chemicals Required:

S.N	Chemicals	Brand	Descriptions
1.	N,N-Dimethyl formamide	Sigma-Aldrich	
2.	Methanol	Merck	Emsure, ACS,ISO, Reag phEur
3.	n-Hexane	Merck	Emsure,ACS
4.	HPTLC plates	Merck	HPTLC-Fertigplatten RP-18 Without fluorescent indicator precoated with concentrating zone 25plates 10×20 cm for nano TLC Concentrating zone 2.5cm ×20 cm
5.	HPTLC Plates Silica gel 60	Merck	Without fluorescent indicator , precoated 100 plates 5×5 cm for nano TLC

3.3 Plant Material

Seedlings of Barley (Hordeum vulgare) were grown in moist vermiculate for upto four days and six hours in growth chamber.The specification is Barley, *Hordeum vulgare L,* cultivar steffi Saatzucht Ackermann & Co, Irlbach. No light was allowed to penetrate the growth chamber. The temperature inside the growth chamber was maintained at 25 degree centrigade.

3.4 Instruments and Equipments:

Fluorescence spectrophotometer:

How to use the instrument:

1. Switch ON the following instruments: fan, cooler, Amherst scientific named instrument, fluorohub(Horib and jobin yvon)

2. Switch on the computer.

3. The lamp is switched ON after 30 minutes. Listen to the first bip and two continuous bip on the processor which is the indication that the system is ready for use.

4. Press program Fluoressence.

5. Press Methods, spectra, Emission ,Load sequentially.

6.Load the sample(around 600 µl in the cuvette and place on the machine . Based on different excitation wavelength find the emission spectrum. Normally the excitation spectrum is initially taken to be 440nm.

7. Based on the emission spectrum find the excitation spectrum. Inorder to find the excitation, we narrow the excitation silt and boarden the emission silt.

8. If the sample concentration in the graph is shown to be more than 1.0×10^{6}, we have to dilute the sample or change the size of the silt so that the concentration is below the value.

3.5 Plant Growth Condition

The model plant used for the experiment to characterize the pigments was *Hordeum vaulagare* L. The seeds were planted on a vermiculate bed (Klein and Mullet 1986) with few modifications by (Eichacker, Soll et al. 1990). The incubator was completely dark. The temperature inside the growth chamber was 25 ˚c. After four days and six hours, the etiolated plants was cut and exposed to the light conditions based on the requirement of the experiment.

3.6 Leaf illumination and dark incubation:

The tray with etiolated leaves were taken out of the incubation chamber and kept in the laminar hood in the dark room at room temperature. Ten leaves were cut from the tray. The cut leaves were kept in the cutting board. Each leaf was cut two centimeters from the top and kept in the cryovile (3 ml containing vile) one at a time. The cryoviles were numbered from Zero complete dark to sixty minutes. It should be noted that in case of Complete Dark sample all the processes of removing leaves from the tray, cutting them and putting them were done without green safelight while other samples were done in the green safe light. The lid of the cryovile was opened and the leaves were illuminated with electronic photographic flash (full flash) by placing the mouth of the vile touching the tip of the light source. All the samples were exposed to a continuous light source of 10 seconds and the respective dark period and placed in the box containing the liquid nitrogen. The detailed table below explains the time of illumination and their dark periods before they were kept in the box containing liquid nitrogen.

Sample number	Name of the sample	Notation on the cryovile	Time of Illumination	Time Dark incubation	Miscellaneous
1.	Complete Dark	0_{CD}	No illumination	No dark incubation	Complete Dark
2.	Zero Dark	0_D	No illumination	No dark Incubation	Under Green Safelight
3.	Zero light	0_L	10 seconds	No Dark incubation	Under Green Safelight
4.	15 seconds	5''	10 seconds	5 seconds	Under Green Safelight
5.	30 seconds	20''	10 seonds	20 seconds	Under Green Safelight
6.	45 seconds	35''	10 seconds	35 seconds	Under green Safelight
7.	1 minute	50''	10 seconds	50 seconds	Under Green Safelight
8.	1 minute 30 seconds	80''	10 seconds	80 seconds	Under Green Safelight
9.	2 minutes	110''	10 seconds	110 seconds	Under Green Safelight
10.	3 minutes	170''	10 seconds	170 seconds	Under Green Safelight
11.	4 minutes	230''	10 seconds	230 seconds	Under Green Safelight
12.	5 minutes	290''	10 seconds	290 seconds	Under Green Safelight

13.	10 minutes	590''	10 seconds	590 seconds	Under Green Safelight
Sample number	Name of the sample	Notation on the cryovile	Time of Illumination	Time Dark incubation	Miscellaneous
14.	20 minutes	20'	10 seconds	1190 seconds	Under green Safelight
15.	40 minutes	40'	10 seconds	2390 seconds	Under Green Safelight
16.	1 hour	60'	10 seconds	3590 Seconds	Under Green Safelight

3.7 Pigment extraction and analysis:

The frozen leaves were crushed using the glass rod which acts like a homogenizer. Three ml of DMF(N,N-Dimethyl form amide) was added to each vile and kept in freezer at four degree centigrade for overnight incubation(Moran and Porath 1980). Two ml of each sample was pipetted in a 2ml eppendorf tube. Each of the two was marked with the annotation and rapped with aluminum foil. All the samples were centrifuged in a centrifuge machine at 8000 g (i.e. 6400 rpm) to remove the unwanted solid particles. The supernatant was removed in new eppendorf tube for further analysis.

Hexane phase extraction:

The supernatant was washed three times with 1ml of *n*-hexane to extract esterified pigments. Non-esterified pigments, protochlorophyllide and chlorophillide, remained in DMF solution. The esterified pigments in the hexane fraction were determined by spectrophotometer.

Chlorophyll concentration estimation:

Using spectrophotometer:

The spectrophotometer used was UV-2401 PC UV-Vis recording spectrophotometer of Shimadzu Company. The baseline for the spectrum observation was chosen between 300-750 nm.

The glass tube used in the spectrophotometer was rinsed with acetone and then with DMF. One ml of the DMF extracted sample was pipetted in the tube. One ml of DMF was used as the reference sample. The absorption was calculated by the spectrophotometer and the concentration was calculated using the equation given by Dr Schoch :

$$\text{Chl a} = 14.15 \times (A_{662} - A_{750}) - 3.34 \times (A_{644} - A_{750}) - 0.05 \times (A_{624} - A_{750}) \text{ (nmol/ml)}$$

$$\text{Chl b} = 25.83 \times (A_{644} - A_{750}) - 6.00 \times (A_{662} - A_{750}) - 1.1 \times (A_{624} - A_{750}) \text{ (nmol/ml)}$$

Spectrofluorometry:

Fluorescence spectra were recorded in Fluorolog-3 spectrofluorometer (with fluorescence software) of the company Horiba Jobin Yvon.

The emission spectra were recorded at excitation and emission silt bandwidths of 6nm. The emission spectra of pigments were recorded from 460 to 750 nm. The excitation wavelength was taken in the range of 300-480nm.

Scanning of the RP-TLC:

The scanning was done in Typhoon Scanner model Typhoon 100-240v 50/60 Hz with the serial number of 1401575 of GE healthcare Bio Sciences AB 75184 Uppsala,Sweden. While scanning the acquisition mode was Fluorescence and it was scanned in 670BP cy5 in normal sensitivity with the pixel size of 200 micron and focal plain is platen.

4.Results:

The pigments of the etiolated leaves and deetiolated leaves were extracted and observed in the RP-18(octadecyl carbon bonded silica) chromatography plate. The pigments present in the etiolated plants were clearly visible in the RP-18 plates. The migration rates of the pigments were calculated. In order to identify the extracted pigments were further run along with the standard chlorophyll pigments and the pigments were compared. Each sample was then analyzed by spectrophotometer. Based on the absorption of the light by the pigments their absorption spectrums were calculated. The samples were further analyzed by fluorescence spectrometer.

Reverse phase chromatography:

We know that the rate of movement of each pigment on a thin layer depends on its relative affinity for the stationary and mobile phases. When polar molecules are present along with the mixture, a polar organic solvent mixed with a small amount of water is recommended. The mobile phase is significantly more polar .The stationary phase is hydrophobic .The stationary phase used in our experiment is a micro porous silica-based material with chemically bonded alkyl chains. The sample containing the pigments which need to be separated was first applied to the stationary phase of the reverse phase chromatography and the mobile phase was allowed to flow past the immobile phase. The pigments were distinctly separated in our RP-18 (octadecyl carbon chain bonded silica) chromatography plate.

The mobile polar phase used was Methanol: acetone: water in the ratio 30:20:1**.**The polarity is given as the dielectric constant .The dielectric constant of methanol is 32.63 . The dielectric constant of acetone is 20.7 and water is 80.3. With the increase in the dielectric constant the polarity increases. That means water is more polar than methanol and acetone. According to the principle of thin layer chromatography non-polar solvent moves non-polar substance. So if we increase the acetone amount than the chlorophyll moves higher.

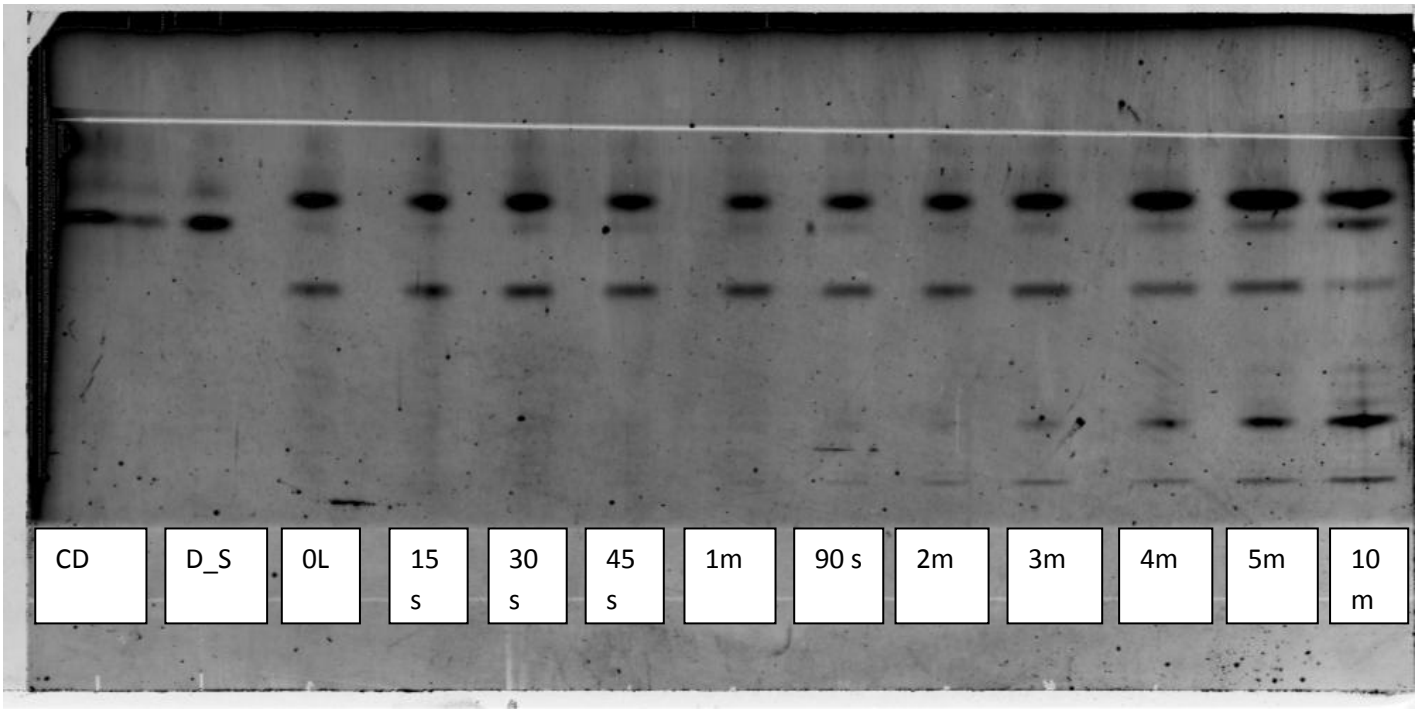


Figure 8i: Reverse Phase Thin layer chromatography

This figure shows the pigments formed when the dark etiolated plant was exposed to the light for ten seconds and was dark incubated for different time intervals till ten minutes of dark incubation. The notations used mean the following: CD(Complete Dark sample),D_S meaning Dark sample carried under green safelight 0_L(0-zero, L-light ,Zero light sample), 15s (s denoting seconds, 10 seconds light exposed and five seconds dark incubated sample), 1m(here 'm' denotes minutes where the sample is 10 seconds light exposed and 50 seconds dark incubated),5m(10 seconds light exposed and 290 seconds dark incubated), 10m (10 seconds light exposed and 590 seconds dark incubated).

It can be seen that there is a pigment in the dark incubated plant sample. This result shows that once the light is exposed to the plant sample for ten seconds there is the formation of two other pigments which is assumed to be Divinyl protochlorophyllide on the top and a chlorophyllide band at the lower position when compared to the first pigment. The intensity of chlorophyllide band decreases with the increase of the dark incubation. The chlorophyll pigment is seen to increase in intensity with the decrease in the intensity of chlorophyllide pigment and it is very intense at ten minute dark incubation. There is also a band at the lowest part of the chromatography which is assumed to be pheophytin.

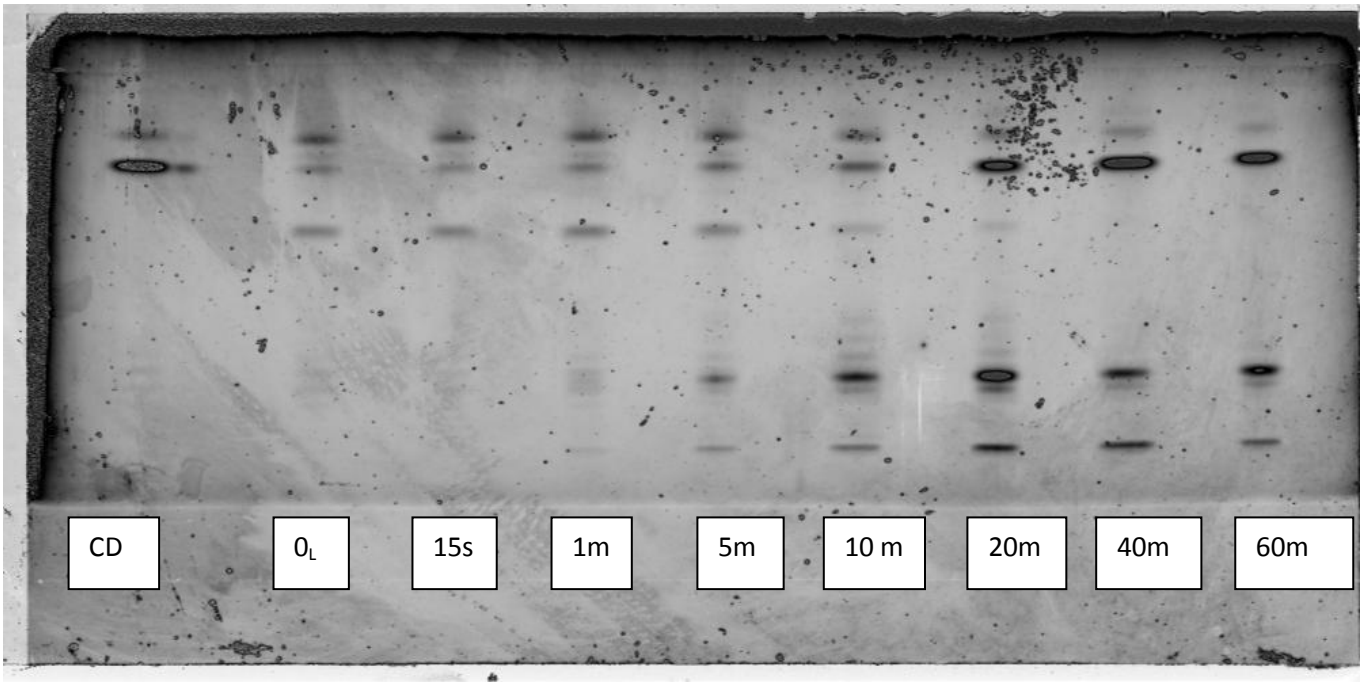


Figure 8ii: Reverse Phase Thin layer chromatography

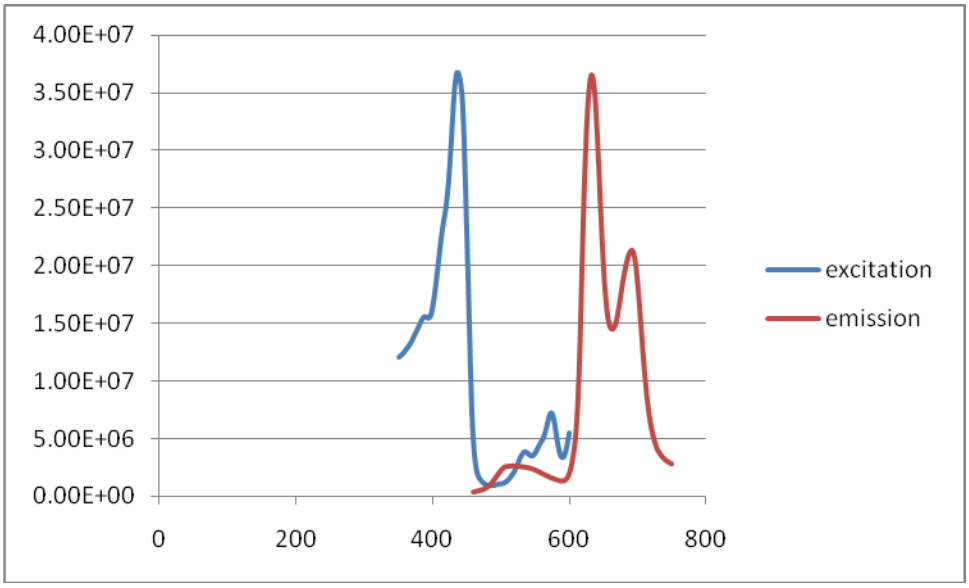
The figure shows the different pigments seen when RP-TLC plate when different samples were run in mobile phase solvent of Methanol:Acetone:HEPES Buffer (30:20:1). The plant was exposed to ten seconds and was kept at different dark incubation upto sixty minutes .The notation used in the figure denotes the following meaning: CD(Complete Dark sample), 0_L(0-zero, L-light ,Zero light sample), 5s (s denoting seconds, 10 seconds light exposed and five seconds dark incubated sample), 1m(here 'm' denotes minutes where the sample is 10 seconds light exposed and 50 seconds dark incubated),5m(10 seconds light exposed and 290 seconds dark incubated), 10m (10 seconds light exposed and 590 seconds dark incubated), 20m (10 seconds light exposed and remaining of 20 minutes is dark incubated),40m(10 seconds light exposed and the remaining of 40 minutes is dark incubated), 60m(10 seconds light exposed to light and remaining of the 60 minutes is dark incubated).

Detection of protochlorophyllide :

The assumption that the pigment formed in the complete dark is protochlorophyllide was validated by observing the absorption spectrum in spectrophotometer. The pigment had a absorption maxima at 624 nm at red band as shown in figure 10 in the spectrophotometric graph. The pigment was further analyzed in fluorescence

spectrophotometer and the maxima excitation spectra is at 435 nm and the emission maxima is at 632 nm and 692 nm as shown in the graph below:

Dark Zero sample:

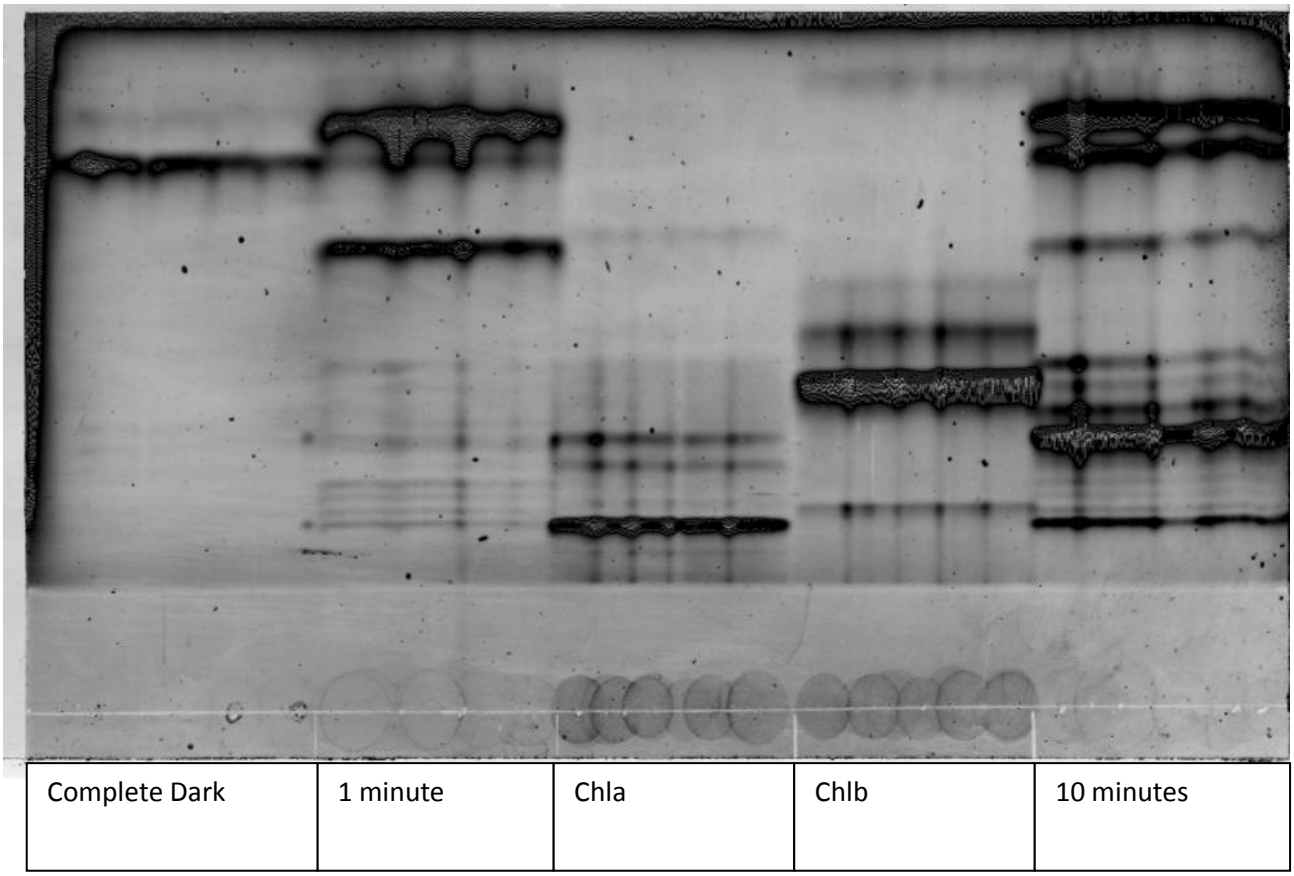


Protochlorophyllide has emission spectrum at 638nm when excited at 440nm..Chlorophyllide also fluorescence at 633 and 638nm. (Hukmani and Tripathy 1992)

The pigment that was extracted in dark is monovinyl protochlorophyllide since it has emission spectrum at 632 nm and the excitation spectrum is 435nm (when emitted at 632nm)

Chlorophyll detection:

The pigment written as chl a and chl b in the figure 8 are the standard chlorophyll sample. By comparision with the standard chlorophyll sample it is clear that ten minutes sample distinctly contain chlorophyll a pigment. But from figure 8i and 8ii it is seen that chlorophyll pigment starts to accumulate from one minute of dark incubation and is very clear at two minutes of dark incubation. However, the chlorophyll b doesnot seem to be present in barley plant.



Complete Dark	1 minute	Chla	Chlb	10 minutes

Figure 9 : Reverse phase Thin layer chromatography

The figure above shows the pigment distribution on the RP-18 plate when the samples were run in mobile phase of Methanol:Acetone : water in the ratio 30:20:1 along with the standard chlorophyll a and b samples. The figure clearly shows that the sample kept in complete dark does not contain chlorophyll a or b. The samples which were initially exposed to ten seconds of flash of light and with one and ten minutes dark incubation contain the chlorophyll a and b. The concentration as seen in the plant sample after one minute is very less as compared to the sample incubated in dark for 10 minutes.

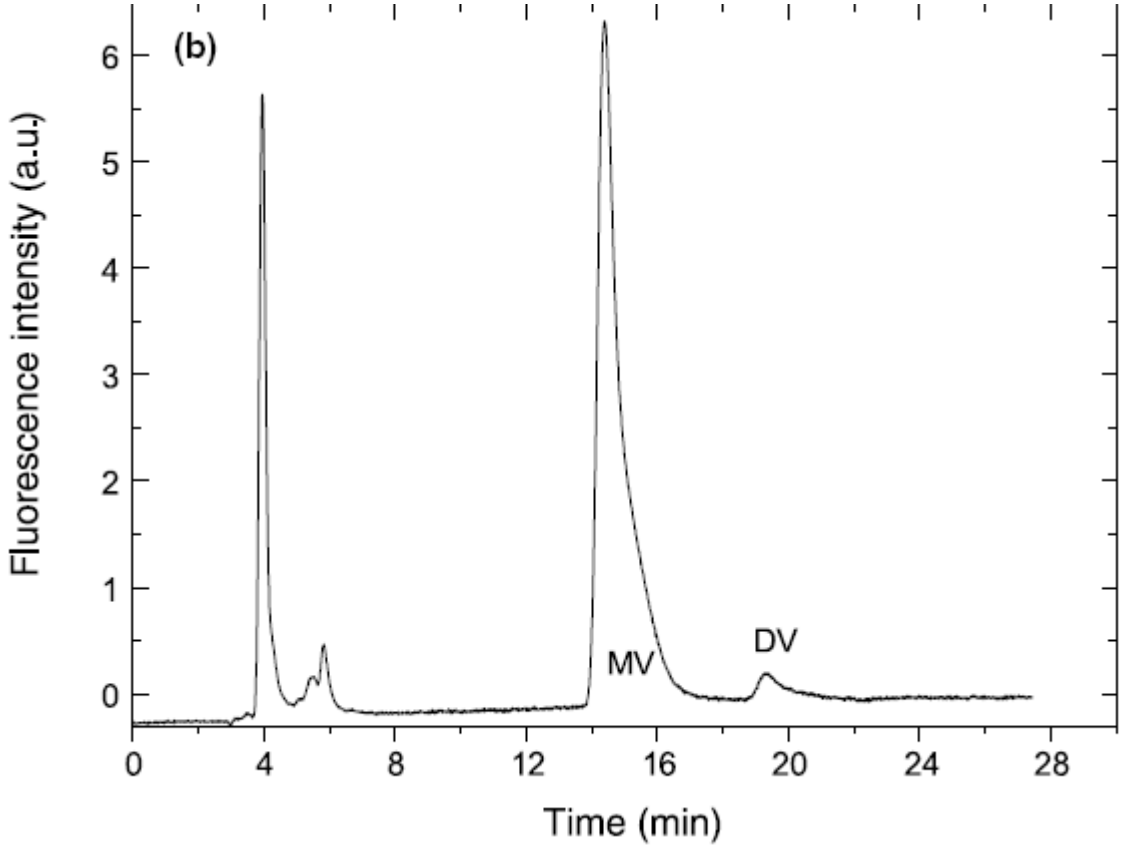
Detection of chlorophyllide and Divinyl Protochlorophyllide:

As the molecules in the mixture move along at different speeds, they are gradually separated. The migration rate of a molecule is constant when using the same mobile – stationary phases, the identity of a particular molecule can be determined from the migration rate. Protochlorophyllide has double bonded C17 and C18 and propionic acid at c17 position. So the top position in the reverse phase chromatography is occupied by the protochlorophyllide. In Monovinyl protochlorophyllide the vinyl group at C8 is

reduced to ethyl group. So the most polar pigment of the chlorophyll biosynthesis is 3,8 Divinyl protochlorophyllide which covers the maximum distance and is seen at the top position of the RP-TLC (figure 8). On the other hand protochlorophyllide is more polar than chlorophyllide. since the double bond present in the ring D of protochlorophyllide is reduced by protochlorophyllide oxidoreductase to form chlorophyllide a. chlorophyllide has propionic acid at the top position of c17 of the molecule. C17 and C18 is reduced. Chlorophyll a has the phytyl group at esterified C17 carbon chain which is the least polar of all the chlorophyll biosynthetic pigments so it is seen to travel least distance in the RP-TLC. Chlorophyll b is more polar than chlorophyll a because of presence of formyl group in C7 of tetrapyrole ring. Both chl a and chl b has reduced c17 and c18 double bond. So, chlorophyll b is more polar than chlorophyll a. so it is seen to be moving ahead in reverse phase thin layer chromatography.

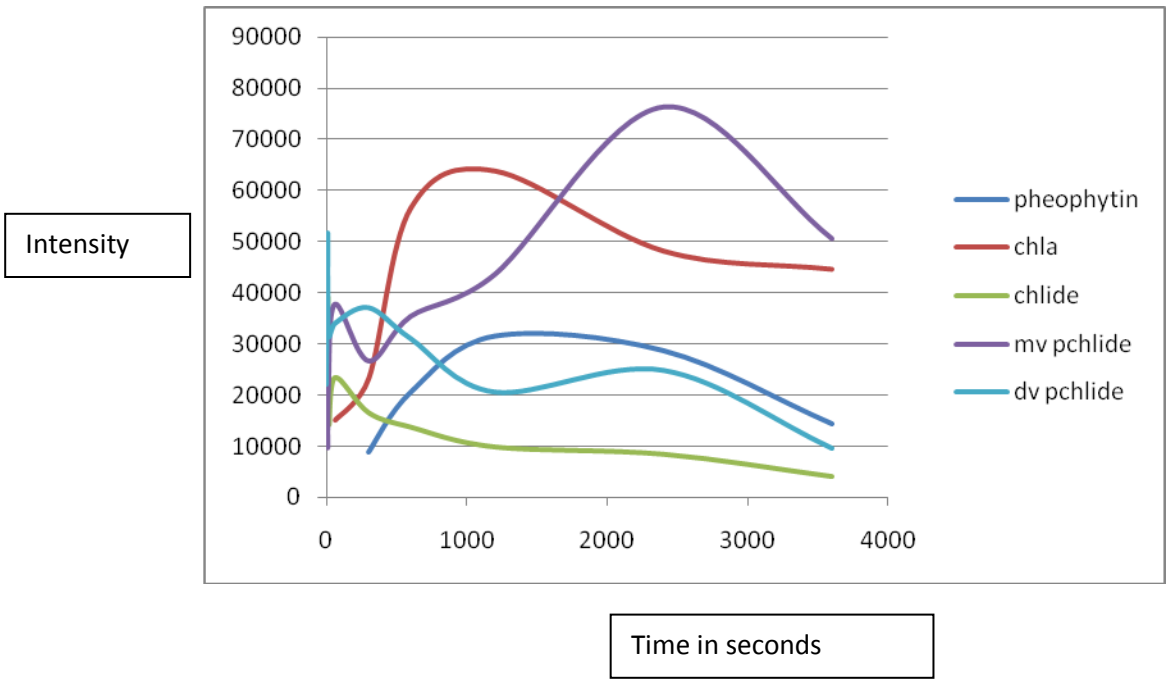
The retention time in RP-HPLC is proportional to the distance travelled in the RP-TLC. The higher the retention time it travels longer distance in RPTLC. In the article(Kruk and MyÅ›liwa-Kurdziel 2004), figure 2 it shows that retention time of DV protochlorophyllide is higher than retention time of MV protochlorophyllide. Similarly the DV protochlorophyllide moves greater distance in RP-TLC than MV protochlorophyllide.So

the higher pigment seen in our result is that of DV protochlorophyllide.



Cited from (Kruk and MyÅ›liwa-Kurdziel 2004)

Similarly for the detection of these pigments, the intensity of the pigments in the figure 8ii was measured and a graph was plotted with the time of incubation in dark and the following graph was obtained by the application of a software called TINA.



Graph 1: Change in the intensities of the pigment at different time intervals.

Figure: Graph showing the intensities of different pigments seen in RP-TLC of figure 8ii at different time intervals. The intensity was calculated using TINA software.

The graph shows that in dark phase, Monovinyl protochlorophyllide is seen at highest intensity. When the plant is exposed to light the Monovinyl protochlorophyllide is reduced drastically and divinyl protochlorophyllide is formed. The Monovinyl protochlorophyllide gains its intensity after twenty minutes dark incubation and it keeps on increasing. The vinyl group at C8 position is proposed to be reduced by 8-vinyl reductase to yield ethyl group to form monovinyl protochlorophyllide. So it can be clearly seen that there is re accumulation of Protochlorophyllide after 20 minutes of dark incubation.

Likewise if you see the figure 8i of this experiment it is clear that complete disappearance of chlorophyllide after 60 minutes and the formation of chlorophyll at this time. If the graph of the chlorophyllide and chlorophyll is seen, they are complementary of each other. The amount of chlorophyll formed is equal to the amount of chlorophyllide lost in the reaction. So it can be said that the decrease in the chlorophyllide is equal to the increase in the chlorophyll content.

The scans of the pigment separations were performed with a too high detector voltage and therefore the low intensity signals are increased and the high intensity have

reached already saturation which makes them not well comparable between the time points.The observation was further clearified by increasing the amount of sample loaded to the RP-TLC. In the figure 10 below , 10 µl of the sample was loaded.

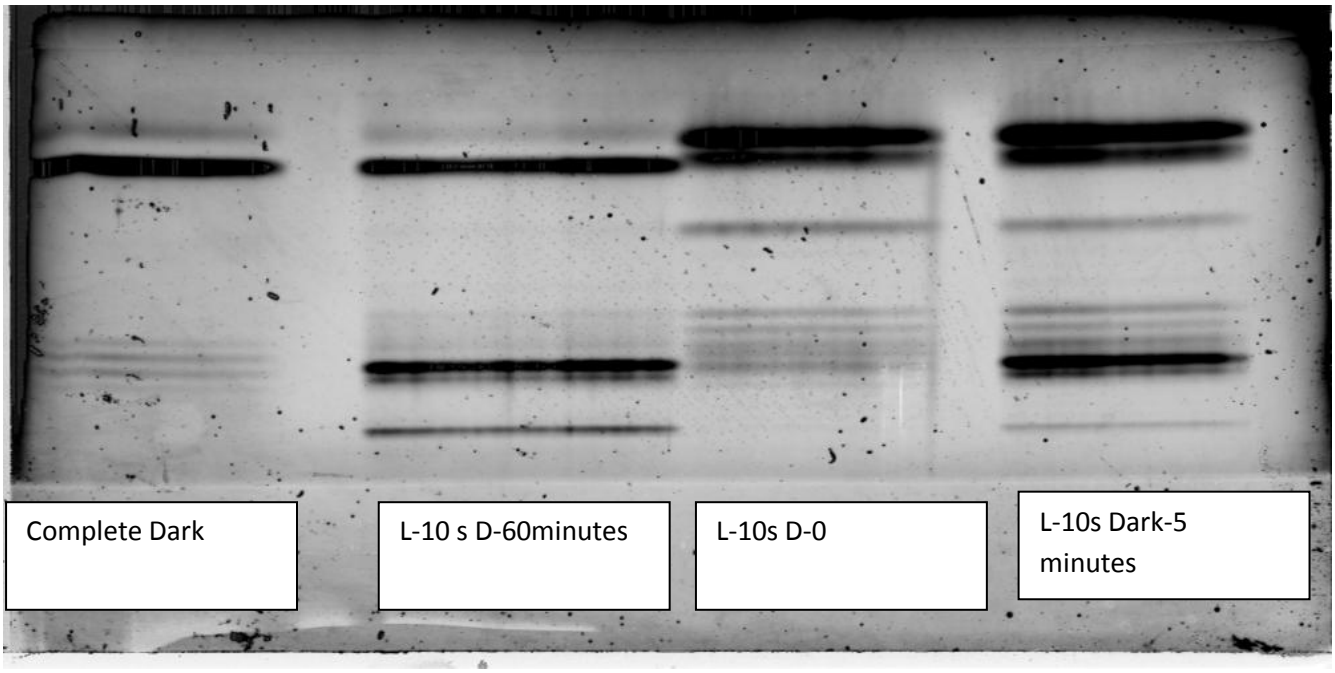


Figure 10: RP-TLC showing the pigments at different time exposure to light and dark incubation.

The figure shows the dark Monovinyl protochlorophyllide in the dark sample. When the plants is exposed to ten seconds of light, the appearance of dark band on top of Monovinyl protochlorophyllide is assumed to be divinyl protochlorophyllide because it is more polar than Monovinyl protochlorophyllide . As we know that divinyl protochlorophyllide is reduced at the C8 vinyl group to yield monovinyl protochlorophyllide by vinyl reductase. So the divinyl protochlorophyllide is more polar then monovinyl protochlorophyllide. So in a RP-TLC, the divinyl protochlorophyllide moves ahead of monovinyl protochlorophyllide so the upper band in above figure may indicate the divinyl protochlorophyllide. Divinyl protochlorophyllide appears more distinct after the light exposure to the etiolated barley leaves.

Spectrophotometric analysis:

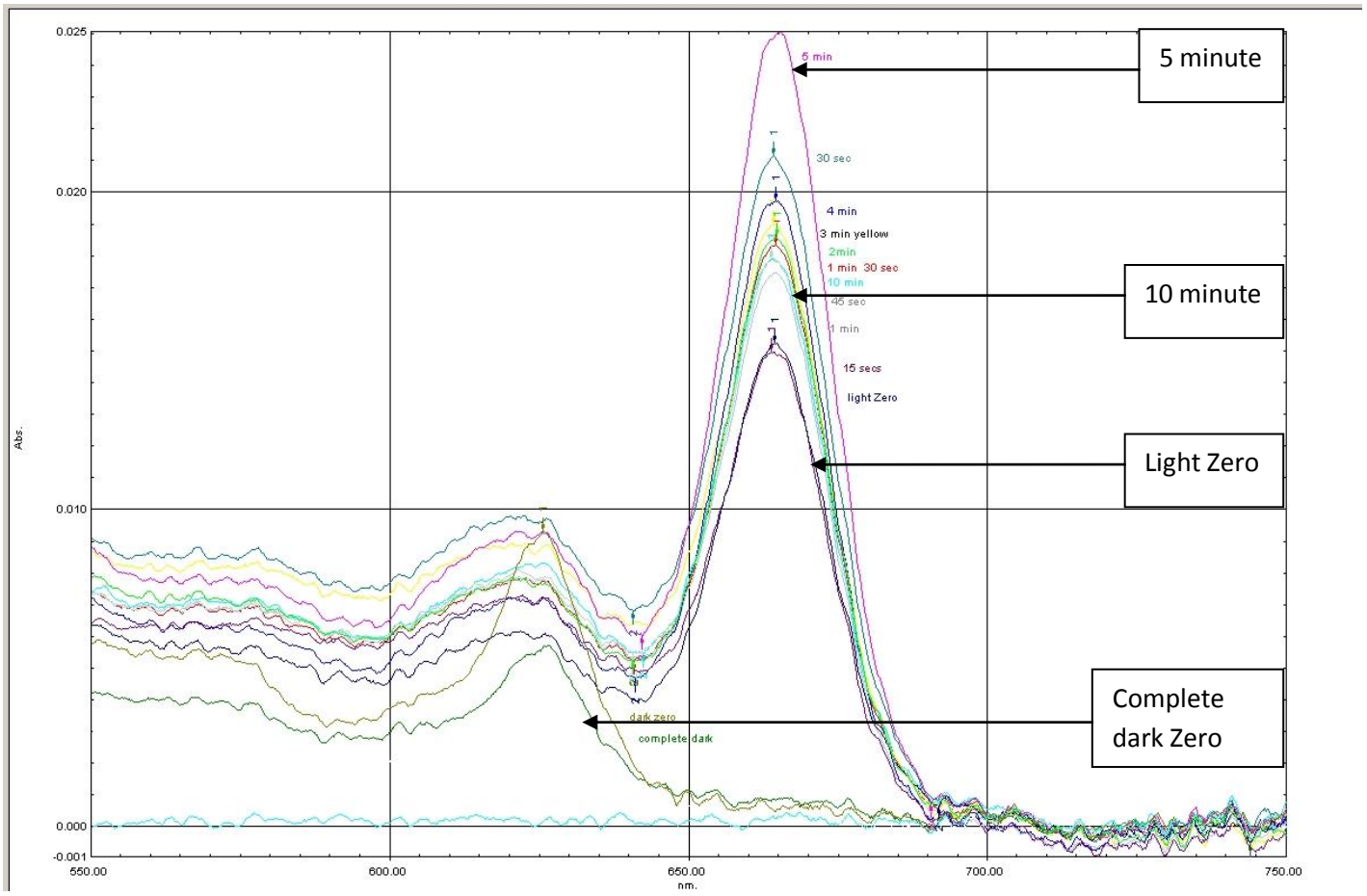


Figure 10: Spectrophotometeric graph showing the absorbance of photosynthetic pigments at wavelength of 550nm to 750nm of the sample that were placed in dark and exposed to light for different time periods.

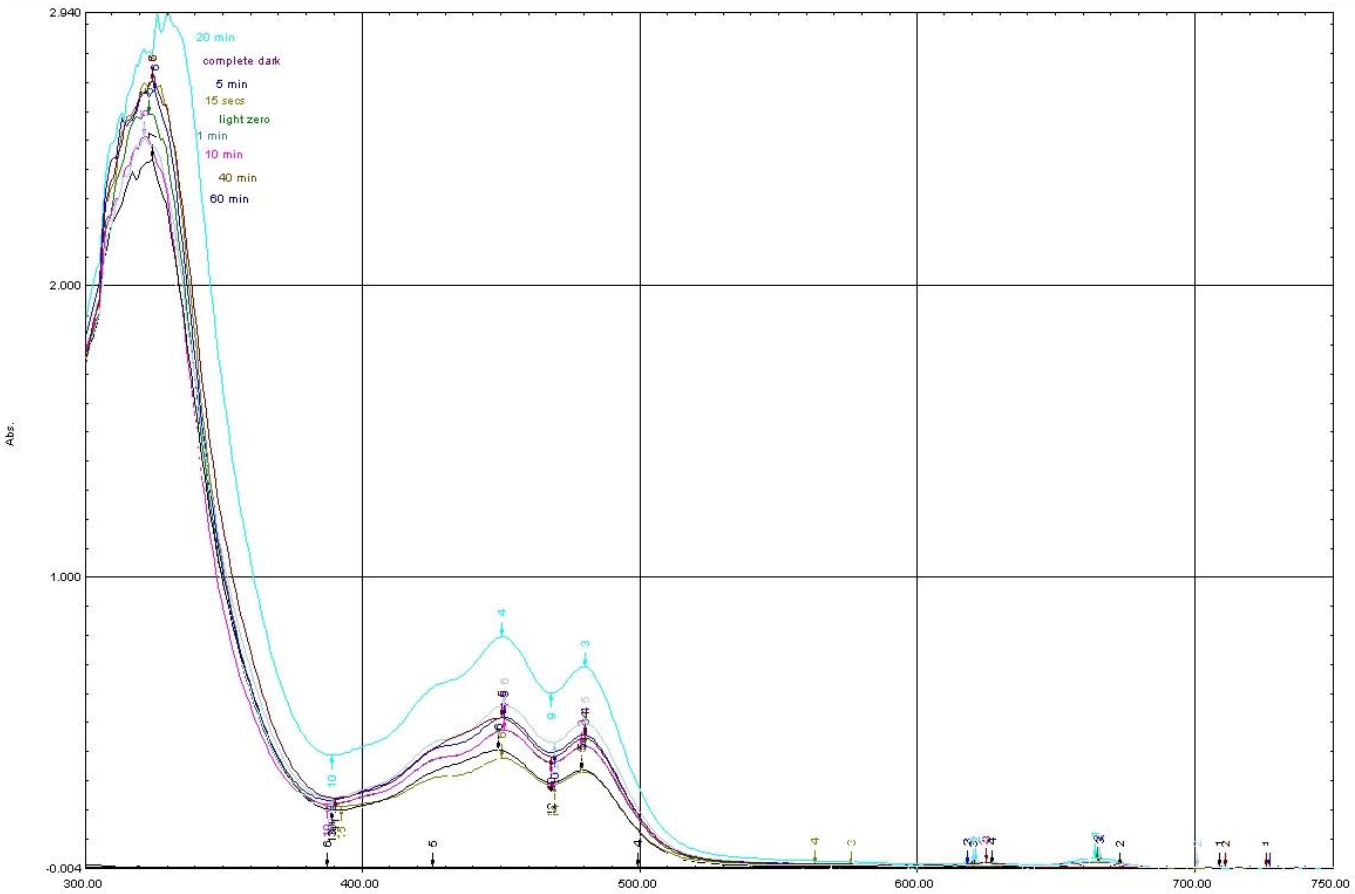


Figure 11: Spectrophotometeric graph showing the absorbance of photosynthetic pigments of the sample from 300nm to 750nm wavelength that were placed in dark and exposed to light for different time periods .

The absorption for complete dark sample was shown to be maximium at 624 nm in one experiment and 625 nm in another experiment at the red band and 323 at the blue band. Monovinyl protochlorophyllide of dark grown barley has absorbance maxima at 431.5 ,569 and 622.5 in diethyl ether(Shioi and Takamiya 1992) and 626.5 in DMF(Moran and Porath 1980). Divinyl protochlorophyllide from nicotinamide enriched culture medium of *R.sphaeroides* has absorbance maxima at 437 573 and 624 .A soret band of MV-pchilde was observed at 435 nm (Shioi and Takamiya 1992). So from the spectrophotometer reading the pigment in complete dark was not clear whether it was monovinyl or divinyl protochlorophyllide but it should be protochlorophyllide.

For all other sample the absorbance maximum was found to be at around 662nm in the red band. The position of the maxima of pure Chl a in DMF is 664.6 and in the blue

region is 433 nm. .(Moran and Porath 1980).There was no uniformity in the absorbance maxima. But it was clear that they contained mostly chlorophyll a . As seen in the graph above in figure 10 , the absorbance of sample dark incubated for five minutes has the highest amount of absorbance and the sample which was not incubated in dark had least absorbance intensity. Thus this absorbance curve showed that when the whole sample is observed in the spectrophotometer, the absorbance is due to the all the pigments present in the sample and they donot show an uniform pattern of absorbance.

Based on the formula:

Chl a = $14.15 \times (A_{662} - A_{750}) - 3.34 \times (A_{644} - A_{750}) - 0.05 \times (A_{624} - A_{750})$ (nmol/ml)

Chl b = $25.83 \times (A_{644} - A_{750}) - 6.00 \times (A_{662} - A_{750}) - 1.1 \times (A_{624} - A_{750})$ (nmol/ml)

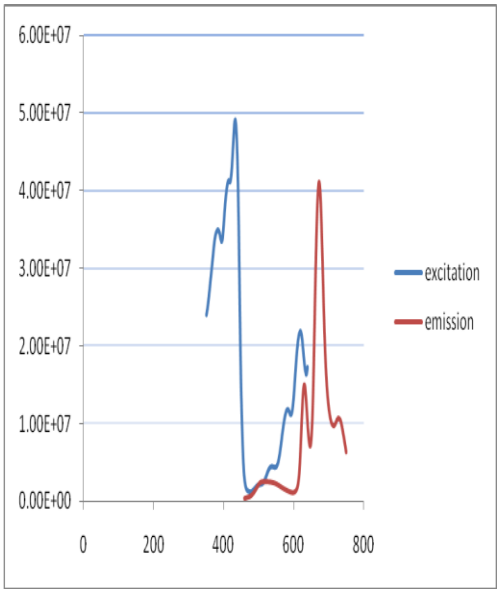
The concentration of chlorophyll a and b were calculated based on the absorbance obtained from spectrophotometer.

S.N	Name of the sample	Chlorophyll a (nmol/ml)	Chlorophyll b (nmol/ml)
1.	Complete dark	0.01	0.014
2.	Dark Zero	0.010	0.0009
3.	Light Zero	0.04	0.0067
4.	15 seconds	0.18	0.03
5.	30 seconds	0.25	0.049
6.	45 seconds	0.22	0.044
7.	60 seconds(1 minute)	0.22	0.018
8.	90 seconds	0.2376	0.012
9.	120 seconds (2minutes)	0.2376	0.01235
10.	180 seconds (3 minutes)	0.23421	0.03708
11.	240 seconds (4 minutes)	0.2518	0.00745
12.	300 seconds (5 minutes)	0.30501	0.00818
13.	600 seconds (10 minutes)	0.25175	0.00635

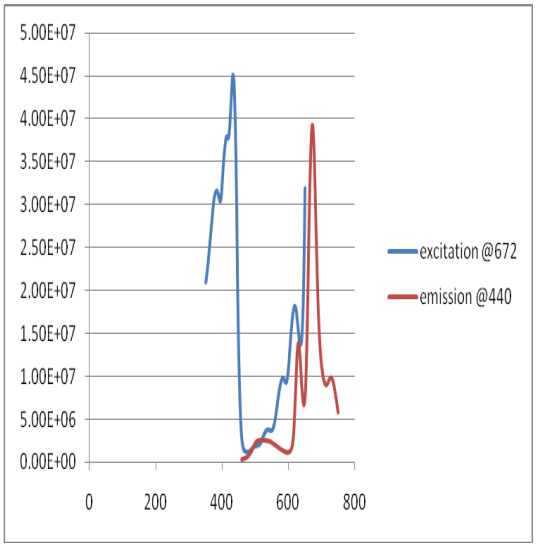
14.	20 minutes	0.39708	0.01124
15.	40 minutes	0.173885	-0.01041
16	60 minutes	0.16611	-0.05387

It is seen that higher amount of chlorophyll a is obtained .The sample was further characterized by measuring the excitation and emission spectrum of sample at different time of dark incubation.

Fluorescence spectrophotometer results:

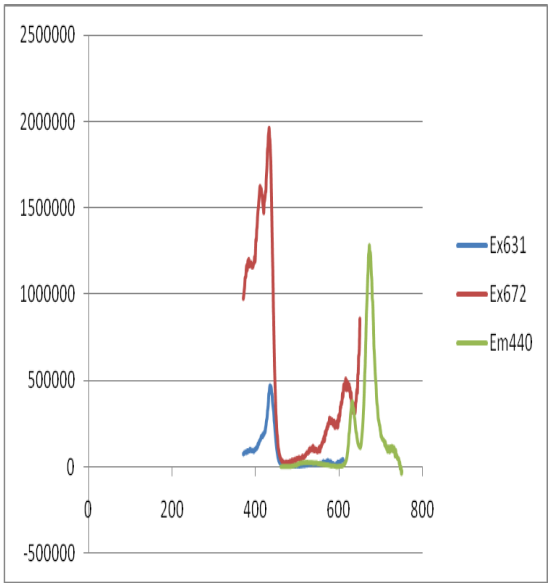


Zero light sample

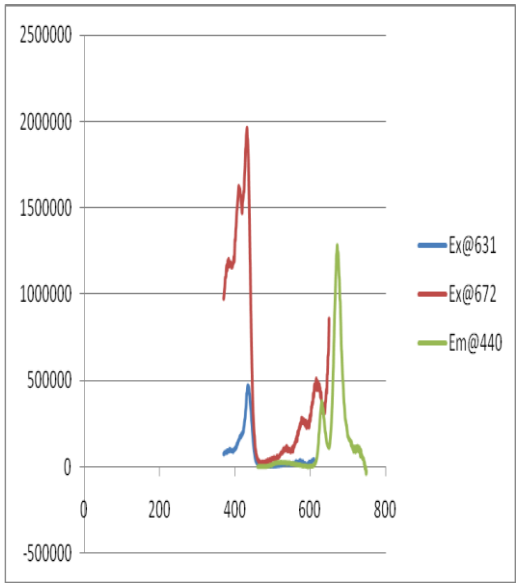


15 seconds sample:

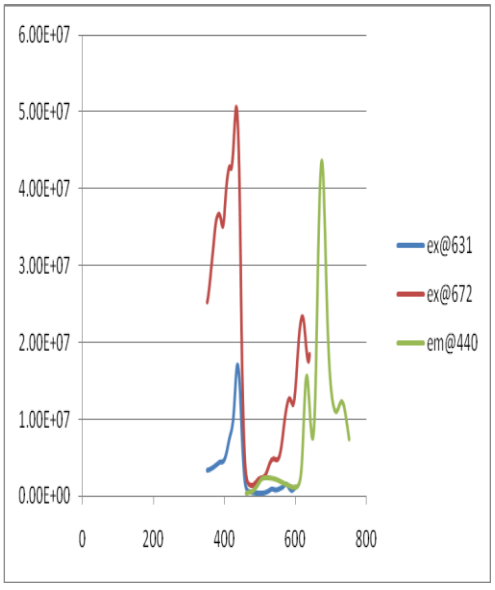
The excitation maxima is at 435 nm and emission maxima is at 631 and 673 nm.



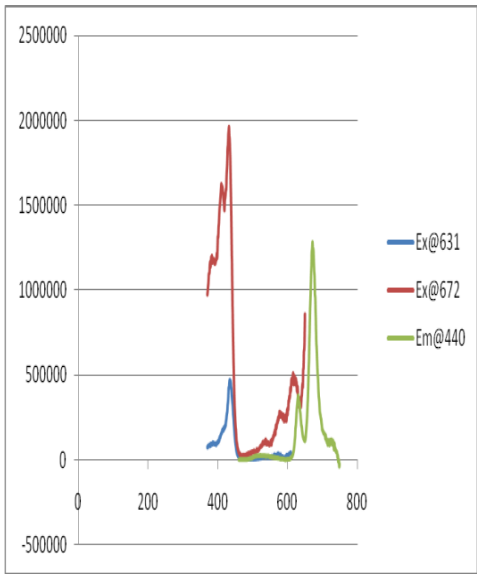
30 seconds sample



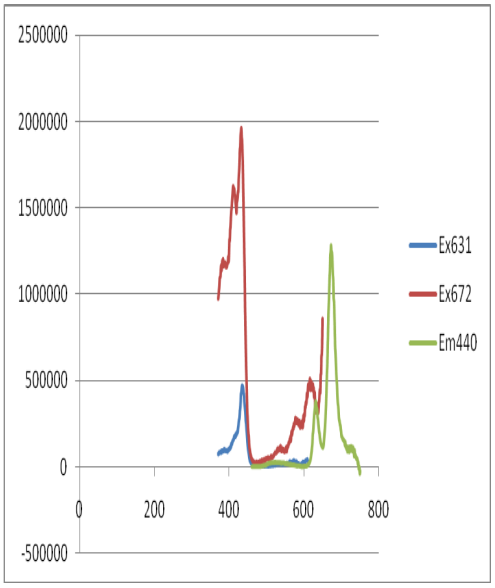
45 seconds sample



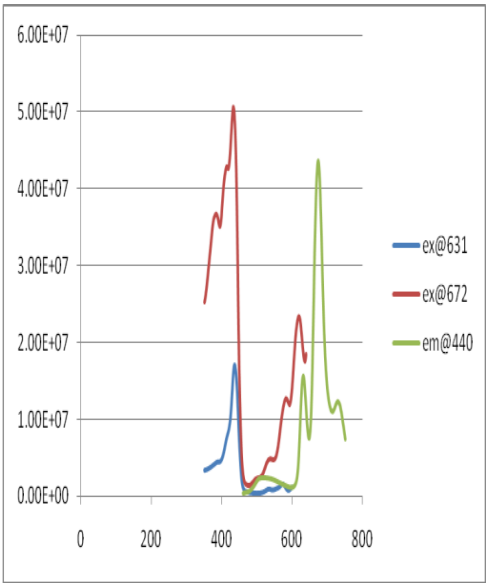
1 minute



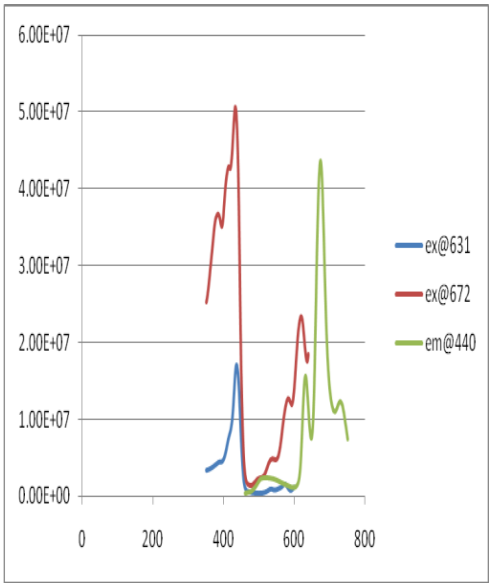
1 minute 30 seconds



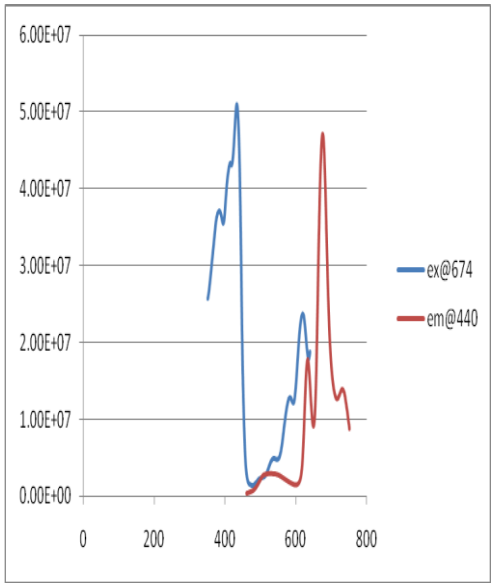
2 minutes



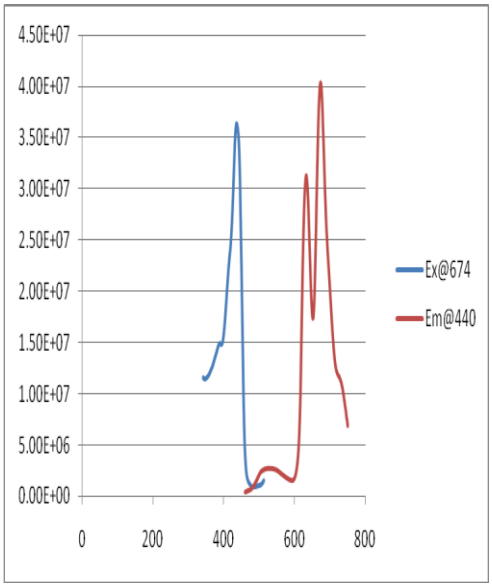
3 minutes



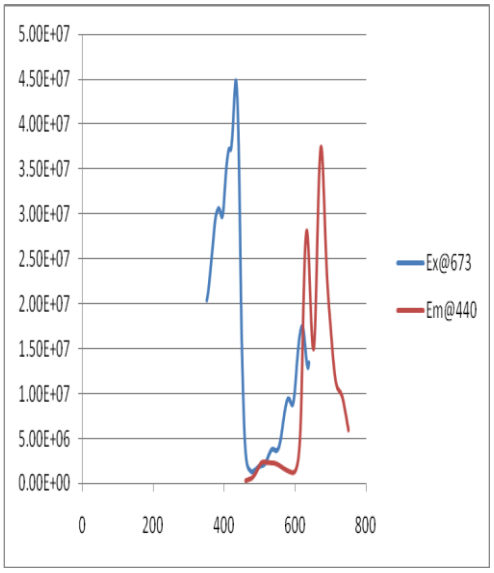
4 minutes



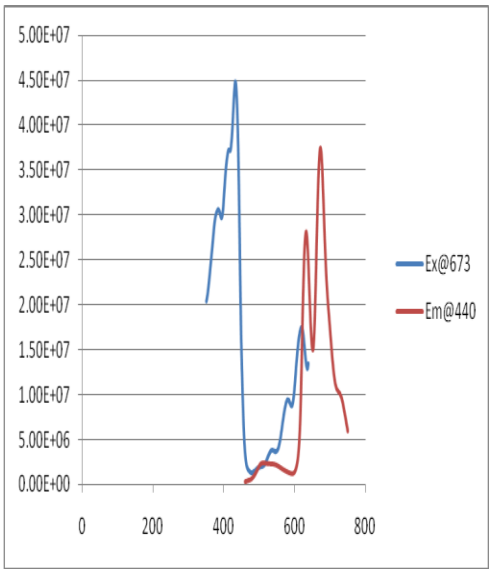
5 minutes

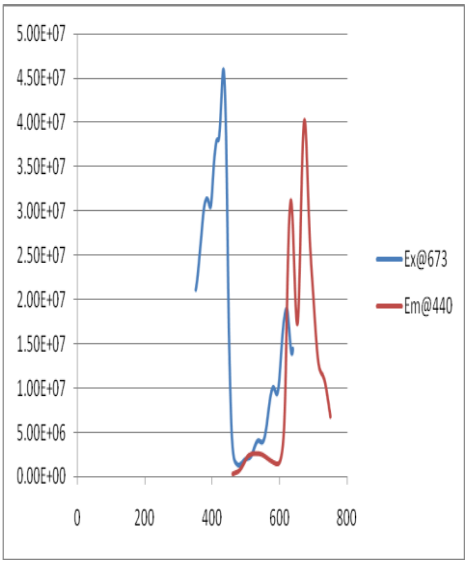


10 minutes



20 minutes





40 minutes 60 minutes

S.N	Name of the sample	Excitation spectrum Maximum (nm)	Emission Spectrum Maximum (nm)
1.	Zero Dark	435	632(Ex at 440)
2.	Zero light	435	631and 673 (Ex at 440)
3.	15 seconds	432 (Em at 631) and 386,412,431(Em at 674)	631 and 674 (Ex at 440)
4	30 seconds	434 (Em at 631) and 383,410,432(Em at 672)	631 and 672 (Ex at 440)
5.	45 seconds	435(Em at 631) and 389, 410 and 431 (Em at 672)	631 and 672 (Ex at 440)
6.	1 minute	435 (Em at 631)and 383,411 and 432(Em at 672)	631 and 672 (Ex at 440)
7.	1 minute 30 seconds	434 (Em at 632) and 386,407, 432 (Em at 672)	632 and 672 (Ex at 435)
8.	2 minutes	436(Em at 632) and 382,413, and 433(Em at 673)	632 and 673 (Ex at 440)
9.	3 minutes	435 (Em at 631) and 385,413, 432	631 and 671 (Ex at 440)

		(Em at 671).	
S.N	Name of the sample	Excitation spectrum	Emission Spectrum
10.	4 minutes	435 (Em at 633) and 387, 409, 431(Em at 672)	633 and 672 (Ex at 440)
11.	5 minutes	435 (Em at 631) and 386, 410, 431(Em at 674).	631 and 674 (Ex at 440)
12.	10 minutes	431(Em at 630) and 385, 407 and 431 (Em at 673)	630 and 674 (Ex at 440)
13.	20 minutes	383, 415 and 432 Em at 673.	632 and 673(Ex at 440)
14.	40 minutes	383, 415 and 432 Em at 673	632 and 673(Ex at 440)
15.	60 minutes	383,415 and 432 Em at 673	632 and 673 (Ex at 440).

Fluorescence spectrophotometer:

The dark sample containing the leaves grown in complete dark showed the maximum peaks of emission spectrum at 632 nm and 692nm and excitation spectrum at 435nm when emission spectrum was taken to be 692nm. ..All other samples showed the maximum peaks of emission spectrum approximately at 631 and 672 nm when excited at 440nm. The samples have maximum peak of excitation spectrum at 435nm when emission spectrum was taken as 631nm.The samples have maximum peak of excitation spectrum at 386, 410 and 431nm when emission spectrum was taken as 672nm. Chlorophyll a has a maxima fluorescence at a wavelength of 673 nm (from Wikipedia) and 726 nm. Divinyl protochlorophyllide has excitation wavelength and emission wavelength : 422 nm and 651 and 712 nm(pchlorophyllide a). In HEAR(293˚K), Proto IX has emission maximum at 633 nm, when excited at 400nm,(E_{400} F_{633}). Under identical conditions Pchlide and chlide, when excited at 440 nm, fluoresce at 638nm (E_{440} F_{638}) and 675nm (E_{440} F_{675}), respectively. (Hukmani and Tripathy 1992). So the

fluoresce emission maxima spectrum at 632 nm could be of protochlorophyllide and the fluoresce emission maxima at 672 may be due to chlorophyllide and chlorophyll. So these bands should be further characterized to identify chlorophyllide and chlorophyll.

5. Discussion:

DMF was very efficient in extracting chlorophyll pigments from barley. Clear bands of protochlorophyllide, chlorophyllide and chlorophyll a were obtained by comparison of the intensity of the different pigments with the standard curve. The absorption spectra of pure Chl a in DMF and in acetone show similarity in the location of the maxima and in the intensity of absorption at the maxima of the red band. DMF can extract pigments from intact tissues, but 80% acetone cannot. In case of tissue in which pigment concentration is low, there is chance of loss of pigments during grinding and centrifuging. The duration of time taken after extraction and examination of the spectrophotometric reading doesnot degrade the absorbance concentration of the pigments.(Moran and Porath 1980) .It is also found that spectrophotometry is not good to analyze the biosynthetic intemediates like protochlorophyllide because they are formed in few picomoles. The spectrofluorometric method is better than spectrophotometetic because it can analyze as low as 1nm concentration of pigments (Hukmani and Tripathy 1992). Based on the results obtained at different dark incubation, the process by which different pigments are formed can be explained based on the biosynthetic pathway of chlorophyll.

Barley plant lack chlorophyll when they grow in absolute dark. They become green when exposed to light. It is due to the formation of chlorophyllide from the protochlorophyllide and due to the formation of chlorophyll with phytol esterified by light independent synthesis.(Domanskii, Rassadina et al. 2003). As seen in the figure 8 I, the plant should be kept at complete darkness for the etioplast to have protochlorophyllide and without any other pigments. The plant should not be exposed even to safe green light because it also induces an appreciable amount of chlorophillide when protochlorophyllide is to be observed. It is stated in literature that it was J.H.C Smith and his group who showed that protochlorophyllide is converted to chlorophyllide when etiolated seedlings are exposed to light (Rudiger 2003). It is also found in the literature that illumination of etiolated plants with flashes of light followed by dark incubation provides enough time for the protochlorophyllide to bind with NADPH and POR which forms large turnover of chlorophyllide(Rudiger 2003). It is very interesting to know the importance of dark incubation for the formation of chlorophyllide. It was found by Rudiger and his colleagues from *in vivo* experiment that large amount of protochlorophyllide was accumulated when etiolated plant was treated with ALA. It was not converted to chlorophyllide in continuous light. When the plants were incubated with dark intervals with each flashes of light the pigment is phototransformable (Rudiger 2003). It is also known that non-photoactive protochlorophyllide which is the protochlorophyllide b doesnot exits in barley (Rudiger 2003).

The appearance of chlorophyllide and disappearance of protochlorophyllide observed in by analyzing the absorbance of spectrophotometer and the emission and excitation spectrum of the fluorescence spectrophotometer is explained in several articles. One of the good explanations about the different shifts in the spectral changes has been given by the experiment of Oliver and Griffiths. In the experiment he found that free protochlorophyllide a has the absorbance maximum at 630 nm. But when protochlorophyllide is bound together with NADPH and POR enzyme, the absorbance shifts to longer wavelength. The complex exists in two forms so it absorbs maximum at 638 nm and 650 nm (Rudiger 2003). When the light is flashed on the plants, there is the photo transformation which leads to the formation of chlorophyllide and $NADP^+$ which has a absorbance maxima at 678 nm. When the $NADP^+$ is reduced to NADPH the absorbance maxima is at 684 nm. But a free chlorophyllide has an absorbance maxima at 672 nm (Rudiger 2003).

It is also revealed that free protochlorophyllide or chlorophyllide is difficult to occur in intact etiolated plants. It is also found by the researchers that there are at least three forms of protochlorophyllide a and more than one form of chlorophyllide a (Rudiger 2003). The shift of the wavelength may indicate the disaggregation of POR complex.

This can be further explained by the biosynthetic process of formation of chlorophyllide from protochlorophyllide in the article by Fujita, 2002. The double bond present in the ring D of protochlorophyllide is reduced by protochlorophyllide oxidoreductase to form chlorophyllide a. The hydrogen used to reduce the protochlorophyllide to chlorophyllide is obtained from NADPH(Rudiger 2003). Protochlorophyllide oxidoreductase functions as a photoreceptor involved in the light dependent reduction of protochlorophyllide. However, this can be done in both light dependent and light independent manner. In the light dependent process, the protochlorophyllide reductase uses the light for the catalysis of the process. The protochlorophyllide oxidoreductase is a single polypeptide enzyme. The protochlorophyllide is bound to the enzyme protochlorophyllide reductase and acts as a cofactor so that it absorbs the light required for the reduction of the molecule. In the dark, light independent process, the protochlorophyllide oxidoreductase is a three subunit enzyme. The reaction mechanism is similar to nitrogenase. The enzyme requires ATP and dithionite also called reduced ferrodoxin for the reaction (Fujita 2002). It is also found that the amount of protochlorophyllide reduction in the dark is proportional to the decrease of ALA synthesis. This clearly suggests that the ALA-synthesizing capacity is adjusted with the level of protochlorophyllide so that the accumulation of photoreactive porphyrins does not occur.(Grimm)

Chlorophyll phytalation is the esterification of alcohol chain at the C17 of the tetrapyrrole molecule at the side chain of the ring D of the chlorophyllide for the formation of chlorophyll. It can take place by two types of reaction. It may involve the formation from

chlide and geranylgeranyldiphosphate (GGPP) or the esterificaton of phytol. It is obtained by the esterification of phytyl pyrophosphate to chlorophyllide. It requires enzymatic reactions catalyzed by the chlorophyll synthase which is encoded by *chlG.* It is also formed by esterification of chlorophyllide with geranylgeranyl pyrophosphate. This is followed by the reduction of three of the four double bonds by geranylgeranyl hydrogenase which is encoded by *chlP*. (Fujita 2002) In the both cases the end product formed by the combination of both enzymes is the Chl-Phy. It is reported that chlorophyll synthase of etiolated plants has a preference for GGPP and chlorophyll synthase of green plants a preference for phy PP. (Domanskii, Rassadina et al. 2003) It has been found that a major difference occurs between the *in vitro* and *in vivo* application of chlide with chl-synthase. According to the article by Domanskii, the whole esterification of phytol in chlorophillide to form chlorophyll-phy is explained in two phases namely, the rapid and main phase. The rapid phase is completed in 15-30 seconds followed by the lag phase which lasted for two minutes and the main phase which last for 30-60 minutes. In the rapid phase 15% of the Chlide was esterified and remaining 85% was esterified within 30-60 minutes. Likewise as shown in the figure 8i of this experiment it is seen that chlorophyll starts to accumulate after two minutes and complete disappearance of chlorophyllide and formation of chlorophyll is seen after 60 minutes. It is also reported that the dark period of at least 10 minutes is required for the rapid esterification of chlorophyllide,(Domanskii, Rassadina et al. 2003) similarly it is very clear from our experiment as shown in the figure 8ii chlorophyllide pigment loses most of it to form chlorophyll after 10 minutes of dark incubation. The esterification of chlide is also explained in terms of different phases. It is reported that GGPP: Chl-GG accumulation takes place at rapid phase that is before two minutes of light flash and the stepwise hydrogenation to Chl-phy occurs after rapid esterification(Domanskii, Rassadina et al. 2003).

This observation was further elaborated in terms of morphological changes that occur in the internal membrane system of etioplast in the paper by Domanskii. It is found that , the etioplasts of the dark grown plants have excessive amount of Paracrystalline prolamellar body (PLB) and have less prothalakoids (PTs) (Domanskii, Rassadina et al. 2003). The internal membrane system of etioplasts consists of PLB and PTs. The size and its proportion depends on the development stage of the plastids (Ryberg and Sundqvist 1991). In the same article it is said that the morphological changes that occurs with the transfer of etiolated plants to light means that a rapid loss of the regular structure of PLBs followed by their dispersal and relocation of POR to the PTs and it occurs in 5 to 30 minutes(Domanskii, Rassadina et al. 2003). It is also found that that size of the PLB get reduced after the plant is exposed to light and kept in dark incubation for 2 to 5 minutes and a large amount of prothyllakoid is formed. (Domanskii, Rassadina et al. 2003). It is also found that there is certain relationship between amount

of POR enzyme, amount of protochlorophyllide accumulated and the size of prolamellar bodies and the extent to which the plant undergo damage when the dark grown plants are taken to the bright light (Rudiger 2003)

The esterification of chlorophyllide is also associated with the spectral blue shift when new chlorophyllide is formed which was done in different experimental conditions (Domanskii, Rassadina et al. 2003). The change in the spectral blue shift has been explained as the displacement of chlorophyllide from the active site of POR which is followed by the disaggregation of PLBs and the chlorophyllide-POR complex is transferred to the PTs.(Domanskii, Rassadina et al. 2003)

It is also reported that esterification of Chlide is temperature dependent and no esterified chl was detected when etiolated leaves were illuminated at 0˚C(Wolff and Price 1957). This experiment was carried out in room temperature and then kept at liquid nitrogen, so the esterification kinetics was not changed.

The protochlorophyllide can be found in the esterified form called the protochlorophyll. The protochlorophyllide can be esterified by one of these alcohols geranylgeraniol, the dihyrdro and tetrahydrocompound to the phytol. It was also demonstrated that protochlorophyll was inert in *in vitro* and *in vivo* conditions. So these protochlorophyll do not convert to chlorophyll. The function of these protochlorophylls are not clear (Rudiger 2003).

Chlorophyll biosynthetic pathway may be from monovinyl to divinyl protochlorophyllide a :

There are two interesting observation from the RP-TLC plate .Firstly the conversion of MV protochlorophyllide to DV protochlorophyllide. Secondly the reappearance of MV protochlorophyllide. From the figure 8 and the graph I it is observed in barley plants at dark have initially maximum amount of MV protochlorophyllide. When the plant is exposed to light, DV protochlorophyllide is formed at high amount. The intensity of DV protochlorophyllide decreases with the formation of more MV protochlorophyllide and also chlorophyllide till ten minutes of dark incubation. After ten minutes of dark incubation there is complete disappearance of chlorophyllide and reappearance of strong intensity of MV protochlorophyllide. Also there is the formation of chlorophyll a. so it may suggest a peculiar characteristic of barley plant where the biosynthetic pathway of chlorophyll can be altered. According to our observation the pathway would be from MV protochlorophyllide to DV protochlorophyllide rather than DV protochlorophyllide to MV protochlorophyllide..

Based on the different experimental conditions all the intermediates between protoporphyrin IX and chlorophyll pigments have been found as monovinyl and divinyl forms (Rudiger 2003). It is known from experiments that angiosperms store a huge amount of protochlorophyllide a in darkness. It has been reported that both monovinyl and divinyl protochlorophyllide are characterized by both chromatographic and spectroscopic methods. It is shown by Rebeiz *et al* that after the plant is exposed to light, the protochlorophyllide before the light did not give the same protochlorophyllide after the light. So the plants were characterized as four different types called the Dark-DV/Light –DV, Dark-MV/Light DV, Dark-DV/Light MV and Dark-MV/Light MV plants. It is also proved that barley is Dark-MV/Light DV plants.(Rudiger 2003)

It has been mentioned that in cucumber both divinyl protochlorophyllide *a* and divinyl chlorophyllide *a* are found in light. It is said that vinyl reductase of the cucumber has higher affinity to divinyl chlorophyllide *a* than for divinyl protochlorophyllide *a*.(Rudiger 2003). DVR reduces 8-vinyl group of tetrapyrrole to the ethyl group in the presence of NADPH which acts as a reductant(Nagata, Tanaka et al. 2005). DVR(Divinyl Reductase) is the only gene that has not been identified for chlorophyll synthesis.(Nagata, Tanaka et al. 2005)A very different case could be hypothesized in barley where the DVR reduces the 3,8 Divinyl protochlorophyllide to form monovinyl protochlorophyllide *a* simultaneously the chlorophyllide *a* is esterified to form chlorophyll *a* in the presence of chlorophyll synthase. However in earlier experiments the proposed theory for the reduction of 8-vinyl group is supposed to be carried before and after the reduction of D-pyrrole ring of tetrapyrrole by protochlorophyllide oxidoreductase.(Nagata, Tanaka et al. 2005) which means after the formation of chlorophyllide. This may be reason why there is disappearance of divinyl protochlorophyllide *a* and chlorophyllide *a* at the same time. The different amount of monovinyl protochlorophyllide accumulated with the increase of dark incubation is explained by *Y.Shioi and K.Takamiya*. It is said that the extent of MV-pchlide accumulation depends on the activity of 8-vinyl reductase, the availability of reductant for the enzyme and the time in darkness. It is suggested that the time of darkness may be necessary for the generation of reductant and/or reductase (Shioi and Takamiya 1992). On the basis of this knowledge, the reappearance of MV protochlorophyllide could be understood to certain extent but the conversion of MV protochlorophyllide to DV protochlorophyllide after illumination is still not clear.

So it is seen that vinyl reductase can reduce early in protochlorophyllide or late in chlorophyllide. It is also seen that hydrogenation of the isoprenoid alcohol before and after esterification. However it is not found in any experiment that function of chlorophyll is altered by the early or late reduction of the substrate.

Further in the experiment:

The experiment can be further carried out by varying the time of light exposure. What changes could occur when the plant kept for 40 minutes is illuminated with light again.

The bands in each time period can be further characterized by fluorescence and spectrophotometry.

Abbreviations

ATP: Adinosine Triphosphate

Chl-phy: Phytylated chlorophyll

DMF: N,N-Dimethylformamide

DVR: Divinyl reductase

GGPP: Geranylgeranyl diphosphate

HEAR-Hexane extracted acetone residue

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

POR: Protochlorophyllide Oxidoreductase

PT: Prothyllakoids

TLC: Thin layer chromatography

Appendix

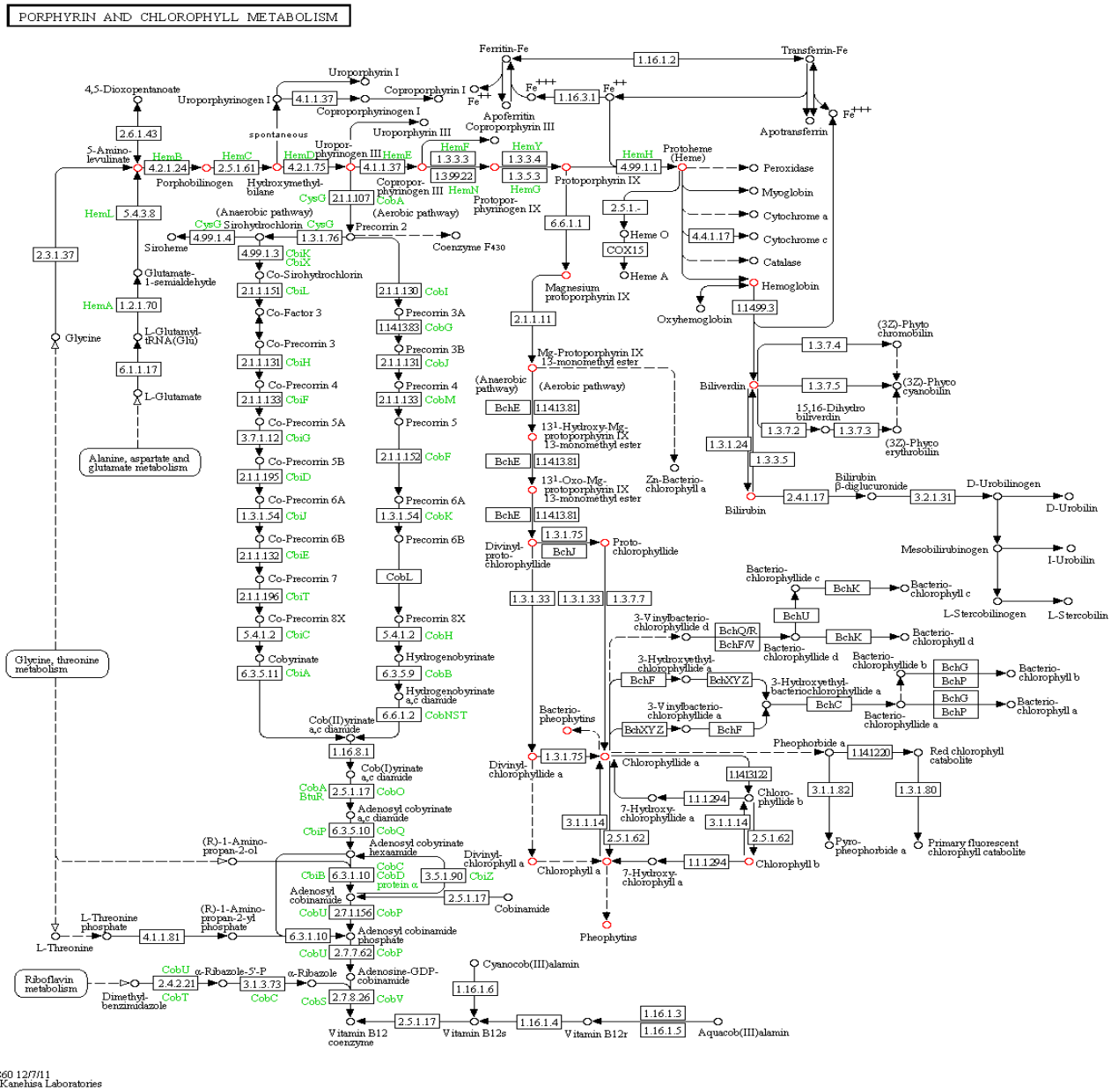
1.Vinyl group:

A **vinyl** compound (formula $-CH=CH_2$) is any organic compound that contains a **vinyl group** (Preferred IUPAC name **ethenyl**),[1] which are derivatives of ethene, $CH_2=CH_2$, with one hydrogen atom replaced with some other group. An industrially important example is vinyl chloride, precursor to PVC, a plastic commonly known as "vinyl".

Vinyl groups are alkene derivatives: Primary alkenes contain vinyl groups. On a carbon skeleton, sp^2-hybridized carbons or positions are often called **vinylic**. Allyls, acrylates and styrenics contain vinyl groups. (A styrenic crosslinker with two vinyl groups is called divinyl benzene).

2.Diterpene, a type of terpene, is an organic compound composed of four isoprene units and has the molecular formula $C_{20}H_{32}$. They derive from geranylgeranyl pyrophosphate. Diterpenes form the basis for biologically important compounds such as retinol, retinal, and phytol.

Chlorophyll biosynthetic pathway


PORPHYRIN AND CHLOROPHYLL METABOLISM
Ferritin-Fe
Transferrin-Fe
Apoferritin
Apotransferrin
Uroporphyrin I
Coproporphyrin I
Uroporphyrinogen I
Coproporphyrinogen I
Uroporphyrin III
Coproporphyrin III
Uroporphyrinogen III
Protoporphyrinogen IX
Protoporphyrin IX
Protoheme (Heme)
Peroxidase
Myoglobin
Cytochrome a
Cytochrome c
Catalase
Hemoglobin
Oxyhemoglobin
Heme
Heme A
COX15
Magnesium protoporphyrin IX
Mg-Protoporphyrin IX 13-monomethyl ester
(Anaerobic pathway)
(Aerobic pathway)
Precorrin 2
Sirohydrochlorin
Siroheme
Coenzyme F430
Co-Sirohydrochlorin
Co-Factor 3
Precorrin 3A
Precorrin 3B
Co-Precorrin 3
Precorrin 4
Co-Precorrin 4
Precorrin 5
Co-Precorrin 5A
Co-Precorrin 5B
Co-Precorrin 6A
Precorrin 6A
Co-Precorrin 6B
Precorrin 6B
Co-Precorrin 7
Co-Precorrin 8X
Precorrin 8X
Cobyrinate
Hydrogenobyrinate
Hydrogenobyrinate a,c diamide
Cob(II)yrinate a,c diamide
Cob(I)yrinate a,c diamide
Adenosyl cobyrinate a,c diamide
Adenosyl cobyrinate hexaamide
Adenosyl cobinamide
Adenosyl cobinamide phosphate
Adenosine-GDP-cobinamide
Cobinamide
Vitamin B12 coenzyme
Vitamin B12s
Vitamin B12r
Aquacob(III)alamin
Cyanocob(III)alamin
Divinyl-proto-chlorophyllide
Proto-chlorophyllide
Divinyl-chlorophyllide a
Chlorophyllide a
Chlorophyll a
Chlorophyll b
7-Hydroxy-chlorophyllide a
7-Hydroxy-chlorophyll a
Pheophytins
Pheophorbide a
Red chlorophyll catabolite
Pyro-pheophorbide a
Primary fluorescent chlorophyll catabolite
Chloro-phyllide b
Bacterio-pheophytins
3-Vinylbacterio-chlorophyllide d
3-Vinylbacterio-chlorophyllide a
3-Hydroxyethyl-chlorophyllide a
3-Hydroxyethyl-bacteriochlorophyllide a
Bacterio-chlorophyllide c
Bacterio-chlorophyllide d
Bacterio-chlorophyllide b
Bacterio-chlorophyllide a
Bacterio-chlorophyll c
Bacterio-chlorophyll d
Bacterio-chlorophyll b
Bacterio-chlorophyll a
Biliverdin
15,16-Dihydro biliverdin
(3Z)-Phyto chromobilin
(3Z)-Phyco cyanobilin
(3Z)-Phyco erythrobilin
Bilirubin
Bilirubin β-diglucuronide
D-Urobilinogen
D-Urobilin
Mesobilirubinogen
I-Urobilin
L-Stercobilinogen
L-Stercobilin
Zn-Bacterio-chlorophyll a
13¹-Hydroxy-Mg-protoporphyrin IX 13-monomethyl ester
13¹-Oxo-Mg-protoporphyrin IX 13-monomethyl ester
5-Amino-levulinate
4,5-Dioxopentanoate
Porphobilinogen
Hydroxymethyl-bilane
Glutamate-1-semialdehyde
L-Glutamyl-tRNA(Glu)
L-Glutamate
Glycine
Alanine, aspartate and glutamate metabolism
Glycine, threonine metabolism
L-Threonine
L-Threonine phosphate
(R)-1-Amino-propan-2-ol
(R)-1-Amino-propan-2-yl phosphate
Riboflavin metabolism
Dimethyl-benzimidazole
α-Ribazole
α-Ribazole-5'-P
spontaneous
(Anaerobic pathway)
(Aerobic pathway)
HemA
HemB
HemC
HemD
HemE
HemF
HemG
HemH
HemL
HemN
HemY
CysG
CobA
CobI
CobG
CobJ
CobM
CobF
CobK
CobL
CobH
CobB
CobNST
CobA
BtuR
CobO
CobP
CobQ
CobC
CobD protein α
CobB
CobZ
CobU
CobS
CobT
CbiA
CbiB
CbiC
CbiD
CbiE
CbiF
CbiG
CbiH
CbiJ
CbiK
CbiL
CbiT
CbiX
BchE
BchJ
BchK
BchU
BchQ/R
BchF/V
BchF
BchXYZ
BchC
BchG
BchP
1.16.1.2
1.16.3.1
2.6.1.43
4.2.1.24
2.5.1.61
4.2.1.75
4.1.1.37
1.3.3.3
1.3.3.4
1.3.5.3
1.3.99.22
4.99.1.1
2.5.1.-
6.6.1.1
4.4.1.17
1.14.99.3
2.1.1.11
1.3.7.4
1.3.7.5
1.3.7.2
1.3.7.3
1.3.1.24
1.3.3.5
2.4.1.17
3.2.1.31
5.4.3.8
4.99.1.4
1.3.1.76
4.99.1.3
2.1.1.151
2.1.1.130
1.14.13.83
2.1.1.131
2.1.1.133
3.7.1.12
2.1.1.152
2.1.1.195
1.3.1.54
2.1.1.132
2.1.1.196
5.4.1.2
6.3.5.11
6.3.5.9
6.6.1.2
1.16.8.1
2.5.1.17
6.3.5.10
6.3.1.10
3.5.1.90
2.5.1.17
2.7.1.156
6.3.1.10
2.7.7.62
2.4.2.21
3.1.3.73
2.7.8.26
1.16.1.6
2.5.1.17
1.16.1.4
1.16.1.3
1.16.1.5
2.3.1.37
1.2.1.70
6.1.1.17
4.1.1.81
1.14.13.81
1.3.1.75
1.3.1.33
1.3.7.7
1.14.13.122
1.14.20
1.1.1.294
3.1.1.14
2.5.1.62
3.1.1.82
1.3.1.80
00860 12/7/11
(c) Kanehisa Laboratories

Spectroscopic photometry

File name: Pawan\eleventh\cd_bd

Complete Darkness sample

S.N	Wavelength	Absorbance
1.	750	0.000
2.	662	0.001
3.	644	0.001
4.	624	0.005

Peak pick

S.N	Wavelength	Absorbance
1.	625	0.014
2.	480	0.443
3.	450	0.513
4.	324	2.703
5.	390	0.237
6.	468	0.379

Concentration of chlorophyll:

Zerolight sample

S.N	Wavelength	Absorbance
1.	750	-0.001
2.	662	0.017

3.	644	0.003
4.	624	0.006

Peak pick

S.N	Wavelength	Absorbance
1.	665	0.018
2.	480	0.417
3.	451	0.472
4.	323	2.590

Filename:Pawan\eleventh\5bd

15 seconds sample

S.N	Wavelength	Absorbance
1.	750	0
2.	662	0.013
3.	644	0.001
4.	624	0.006

S.N	Wavelength	Absorbance
1.	665	0.018
2.	480	0.417
3.	451	0.472
4.	323	2.590

Filename: Pawan\bd\60bd

After one minute

S.N	Wavelength	Absorbance
1.	750	-0.001
2.	662	0.020
3.	644	0.006
4.	624	0.010

Peak pick

S.N	Wavelength	Absorbance
1.	666	0.021
2.	480	0.497
3.	451	0.558
4.	321	2.508

Filename: Eleventh\300bd

After five minutes

S.N	Wavelength	Absorbance
1.	750	-0.001
2.	662	0.020
3.	644	0.006
4.	624	0.008

Peak Pick:

S.N	Wavelength	Absorbance
1.	666	0.021
2.	480	0.454
3.	451	0.516
4.	325	2.668
5.	469	0.394

File name: Eleventh\600bd

After Ten minutes

S.N	Wavelength	Absorbance
1.	750	0.000
2.	662	0.019
3.	644	0.005
4.	624	0.008

Peak pick

S.N	Wavelength	Absorbance
1.	666	0.020
2.	479	0.416
3.	451	0.472
4.	321	2.514
5.	468	0.360

File name: Eleventh\20minutes

S.N	Wavelength	Absorbance
1.	750	0.000
2.	662	0.030
3.	644	0.008
4.	624	0.014

Peak pick

S.N	Wavelength	Absorbance
1.	664	0.030
2.	480	0.690
3.	450	0.793
4.	330	2.940

After 40 minutes

Filename: 40min_bd

S.N	Wavelength	Absorbance
1.	750	0.000
2.	662	0.013
3.	644	0.003
4.	624	0.009

Peak pick

S.N	Wavelength	Absorbance
1.	479	0.289
2.	449	0.020
3.	321	2.360

After 60 minutes

Filename:60min_bd

S.N	Wavelength	Absorbance
1.	750	0.000
2.	662	0.012

3.	644	0.001
4.	624	0.007

Peak pick

S.N	Wavelength	Absorbance
1.	479	0.333
2.	449	0.402
3.	324	2.437

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