THE FORMATION OF A MONOVINYL-FROM A DIVINYL-TETRAPYRROLE IN THE BIOSYNTHETIC PATHWAY OF CHLOROPHYLL *a*.

by

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THE FORMATION OF A MONOVINYL-FROM A DIVINYL-TETRAPYRROLE IN THE BIOSYNTHETIC PATHWAY OF CHLOROPHYLL a.

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ABSTRACT

THE FORMATION OF A MONOVINYL- FROM A DIVINYL- TETRAPYRROLE IN THE BIOSYNTHETIC PATHWAY OF CHLOROPHYLL *a*.

Many angiosperms cannot form chlorophyll *a* when they are grown in the dark, and are said to be "etiolated". Under these conditions, a chlorophyll *a* precursor, protochlorophyllide, accumulates in the internal membranes of chloroplast precursors called etioplasts. The protochlorophyllide which accumulates in the etioplasts of certain plants (*eg.* wheat) has a single vinyl group in the 2-position, and is termed "monovinylprotochlorophyllide". These plants, therefore, are said to employ predominantly a "monovinyl" pathway for chlorophyll *a* synthesis. Other plants (*eg.* cucumber) are thought to synthesize chlorophyll *a* predominantly by a "divinyl" pathway when grown in the dark because the protochlorophyllide pool which accumulates contains relatively more of a 2,4-divinyl derivative of protochlorophyllide (termed "divinylprotochlorophyllide"). The key to the regulation of the relative amounts of intermediates in the monovinyl and divinyl pathways in higher plants may, therefore, be the enzyme responsible for the reduction of the 4-vinyl group of one or more of the intermediates in the biosynthetic pathway. Termed the "4-vinyl reductase", this enzyme has been the subject of the present investigation. The reduction of the 4-vinyl group of both divinylprotochlorophyllide and divinylchlorophyllide was studied in etiolated cucumber and etiolated wheat by fluorescence spectroscopy and HPLC analysis. A new solvent system was developed for the separation of the divinyl- and monovinyl - derivatives of both protochlorophyllide and chlorophyllide during HPLC analysis in powdered polyethylene columns. By the use of this method, evidence has been obtained

that divinylchlorophyllide and NADPH are the true substrates for the 4-vinyl reductase of etiolated cucumber cotyledons. The studies also correlated the formation of a monovinyl- from a divinyl-tetrapyrrole with the incorporation of tritium from stereospecifically labeled nicotinamide coenzymes, the 4R- and 4S-isomers of [^{3}H]NADPH. The results indicated that it was likely that the [4R-^{3}H]NADPH isomer was used by the 4-vinyl reductase of cucumber. The reduction of divinylchlorophyllide to monovinylchlorophyllide may not have been direct, however, and may have proceeded through an intermediate of unknown structure. Discrepancies observed between the results of analyses by fluorescence spectroscopy and HPLC are discussed.

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I would also like to thank Mr. M. Yang for introducing me to HPLC-technigues and fellow members from Dr. Richards group for their friendship.

For their emotional support, many thanks to my family.

DEDICATION

To my son and my beloved family

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURES

ABBREVIATIONS

ADH	alcohol dehydrogenase
Bchl	bacteriochlorophyll
BSA	bovim serum albumin
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
Chl	chlorophyll
DV	divinyl
DVC	divinylchlorophyllide
DVP	divinylprotochlorophyllide
EDTA	ethylenediaminetetraacetic acid
GDH	glucose dehydrogenase
GSH	glutathione, reduced
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
MgP	magnesium protoporphyrin
MgPME	magnesium protoporphyrin monomethyl ester
MV	monovinyl
MVC	monovinylchlorophyllide
MVP	monovinylprotochlorophyllide
PCR	protochlorophyllide reductase
4VR	4-vinyl reductase
TES	N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid
TRICINE	N-tris [hydroxymethyl] methyl glycine
TRIS-HCl	Tris (hydroxymethyl) amino methane-HCl

CHAPTER I

INTRODUCTION

1.1. INTRODUCTION.

The main objective of my thesis project was to gain an understanding of the enzyme catalyzing a latter - stage reaction of the biosynthetic pathway of chlorophyll *a* in plants. The main project was to demonstrate the reduction of a divinyl-to a monovinyl-tetrapyrrole by using either endogenous or exogenous coenzymes (NADPH, NADH) and to correlate the formation of a monovinyl- from a divinyl-tetrapyrrole with the incorporation of tritium from the stereospecifically labeled 4R- and 4S-isomers of [^{3}H]NADPH.

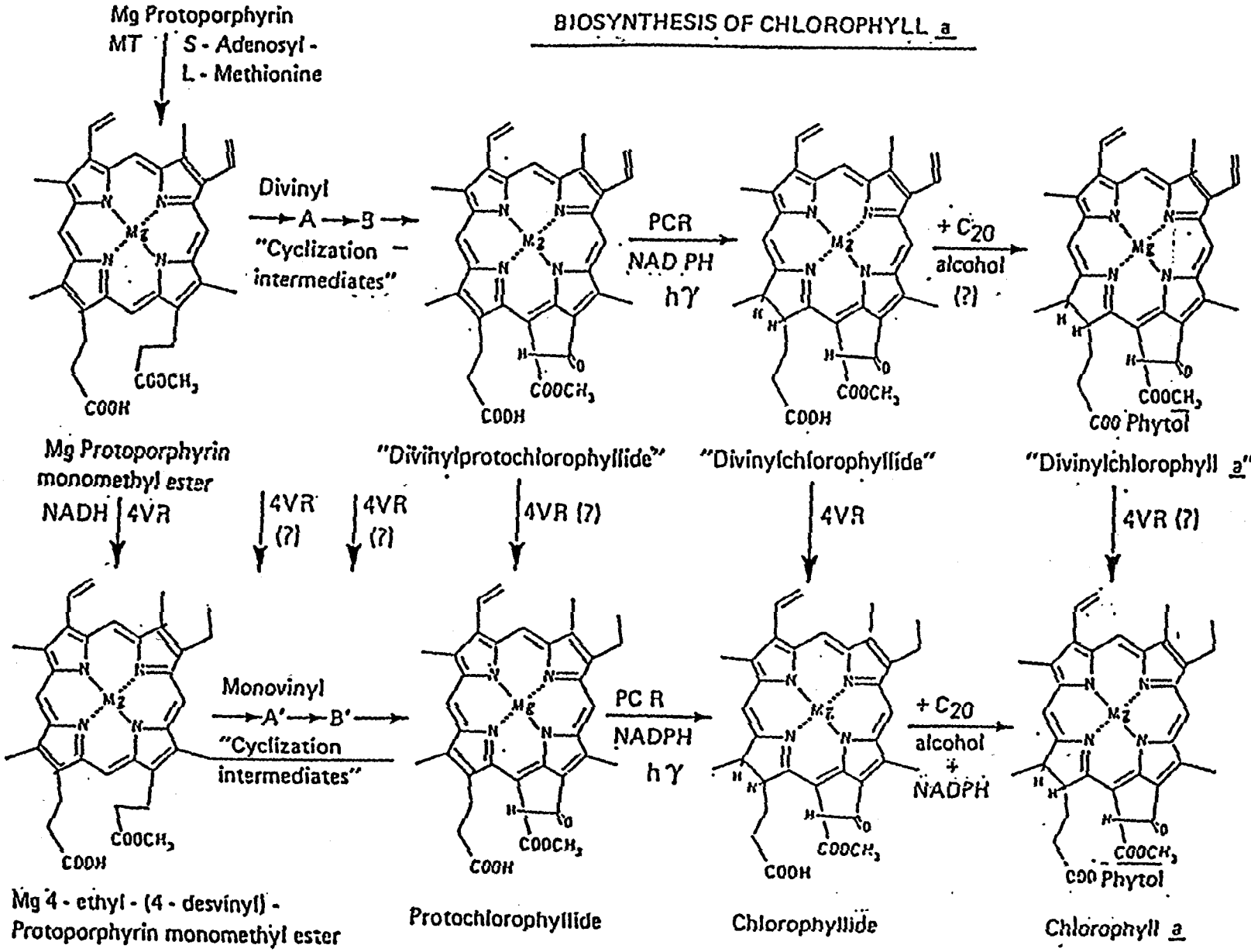
The magnesium branch of tetrapyrrole biosynthesis is commonly represented (1) as a linear sequence of reactions leading from protoporphyrin IX *via* magnesium protoporphyrin IX (MgP) to chlorophyll *a* (Chl *a*). In this pathway divinylprotochlorophyllide (DVP) is derived from magnesium protoporphyrin IX monomethyl ester (MgPME) by an oxidative cyclization reaction sequence. The enzyme system which is responsible for this conversion is MgPME (oxidative) cyclase, and its activity has been studied in intact developing chloroplasts by Castelfranco and coworkers (2-5).

Rebeiz and coworkers (6) have provided a considerable amount of evidence that there are parallel pathways of Chl *a* synthesis in plants. The two major pathways involve monocarboxylic acid intermediates with vinyl substituents in the porphyrin ring at either the 2-position only (monovinyl or MV pathway), or both the 2- and 4-positions (divinyl or DV pathway). The two major pathways are shown in Fig1.1.1. Carey & Rebeiz (7) found that the relative proportion of the two pathways were quite dependent upon the plant species examined and upon the light regime given to the plant. Some plant species (*eg.*cucumber) form

Fig 1.1.1

Monovinyl and divinyl pathways of the latter stage biosynthesis of chlorophyll *a*.

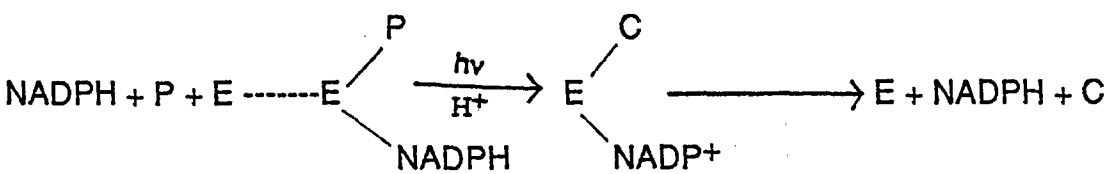
BIOSYNTHESIS OF CHLOROPHYLL a



mainly DVP when growing in the dark, especially when given brief light flashes (8). Others (*eg.*wheat) accumulate mainly normal (monovinyl) protochlorophyllide (MVP) when growing in the dark (7).

The enzyme which reduces the 4-vinyl group to a 4-ethyl group has been termed the 4-vinyl reductase (4VR), and was first detected by Ellsworth & Hsing (9, 10). They reported that the enzyme used NADH (but not NADPH) to reduce MgPME to its monovinyl derivative in etiolated wheat seedlings , but it would not reduce protoporphyrin IX , MgP, or DVP. However, in some plants it may act at a later stage of the pathway and have a major role in determining the relative proportion of intermediates in the MV and DV pools.

In the case of cucumber, Duggan & Rebeiz (8) have demonstrated the 4-vinyl reduction of divinylchlorophyllide (DVC) by using fluorescence spectroscopy. They found that 4-vinyl reduction occurred *only* during a brief period following the photoconversion of accumulated DVP to DVC by the light-requiring enzyme, NADPH: protochlorophyllide oxidoreductase (PCR). PCR has been extensively characterised by Griffiths (11), and has been found to be able to reduce both DVP and MVP .There is evidence that PCR functions by forming a photoactive ternary complex with its two substrates, protochlorophyllide (P) and NADPH (12, 13) and the illumination of such a complex results in hydrogen transfer from NADPH to the porphyrin (13) followed by dissociation of the oxidised nucleotide and chlorophyllide (C) from the enzyme (E):



Duggan & Rebeiz (8) were unable to determine the coenzyme requirement for the 4VR during the reduction of DVC in cucumber cotyledons, however.

Richards and coworkers (14, 15) have tried to use stereospecifically tritium-labeled nicotinamide coenzymes [4R-^{3}H] and [4S-^{3}H]NAD(P)H) during assays for the 4VR, but only very small amounts of tritium were found to be incorporated. Therefore, it was necessary to develop an independent assay (other than radioactive incorporation) for the study of the properties of the 4VR. Hence, the first priority of the present research project was to develop the techniques for the quantitative determination of the relative amount of MV and DV tetrapyrroles. Two major techniques were investigated as summarised in the following section.

1.2. TECHNIQUES FOR DETERMINATION OF THE RELATIVE AMOUNTS OF THE MONOVINYL AND DIVINYL DERIVATIVES IN THE PROTOCHLOROPHYLLIDE AND CHLOROPHYLLIDE POOLS.

1.2.1. *Fluorescence spectroscopy*

Since only small amounts of MV and DV tetrapyrroles accumulate in greening tissue of plants, it was mandatory to develop an analytical method that achieves:
a) high sensitivity (for detection of small amounts of tetrapyrroles)
b) high resolution (for distinguishing MV and DV tetrapyrroles).

Spectrofluorometry provides both of these features. The methodology for the determination of small amounts of tetrapyrroles at 293^{o}K was first described by Bazzaz & Rebeiz (16). It has been shown that the protochlorophyllide pool of etiolated tissue consists of MVP (E437, F625) and of DVP (E443, F625) (17, 18). [E and F refer to the Soret excitation and fluorescence emission maxima of the various tetrapyrroles at 77^{o}K in ether]. Fluorescence spectroscopy has been very successful for the detection and quantitative determination of very small amount of tetrapypyrroles in unpurified mixtures (16, 19, 20, 21, 22, 23,). Tripathy & Rebeiz (21) have described the determination of the relative amounts of any two closely related fluorescent compounds which can be distinguished by spectrofluorometry at 77^{o}K but not at 293^{o}K. They demonstrated a generalized equation for the calculation of the net fluorescence excitation signal of a mixture of MVP and DVP. If (E437, F625) and (E451, F625) represents the amplitude of the fluorescence excitation spectrum at the E wavelength when emission is monitored at the F wavelength, the equations are:

MVP = 1.060 (E437, F625) - 0.964 (E451,F625)

DVP = 1.061 (E451, F625) - 0.068 (E437, F625)

Wu & Rebeiz (22) have recently reported simultaneuous equations for the determination of the relative amounts of MVC and DVC in an unpurified mixture. If (E447, F674) and (E458, F674) represent the amplitude of the fluorescence excitation spectrum at the E wavelength when it is monitored at the F wavelength,

the equations are:

MVC = 1.0205 (E447, F674) - 0.3749 (E458, F674)

DVC = 1.0205 (E458, F674) - 0.0557 (E447, F674)

The equations for the quantitative determination of the relative amounts of MV- and DV-derivatives of the protochlorophyllide and chlorophyllide pools are likely to be very useful for the study of the intermediary metabolism of the MV and DV pathways of Chl *a* biosynthesis in plants. But even under the most ideal conditions it is difficult to determine precisely the relative amounts of MV- and DV- protoporphyrin. This is because the excitation of MV- and DV-protoporphyrin are not that far apart. The chemical structures of MV- and DV-protochlorophyllide (24, 25, 26) and MV- and DV-chlorophyllides (18, 26, 27) were confirmed by NMR and fast atom bombardment mass spectroscopic analyses. Table 1.2.1 gives the published absorption and fluorescence maxima of the pigments in the protochlorophyllide and chlorophyllide pools.

1.2.2. *High performance liquid chromatography*

The analyses of both MV and DV forms of chlorophyllide and protochlorophyllide have been accomplished by fluorescence spectroscopy (21, 22), but a reliable separation procedure of these compounds had not been fully developed at the time of their work. Hanamoto and Castelfranco (28) had earlier reported the separation of both MV- from DV-protochlorophyllide and MV- from

TABLE 1.2.1. The published absorption and fluorescence maxima of MV- and DV-protochlorophyllide and chlorophyllide.

TETRAPYRROLES	ABSORPTION [nm] λ max in ether at room temperature	FLUORESCENCE [nm] in ether at 77°K
MVP	432, 469, 570, 623 (11)	(E437, F625) (19)
DVP (from *Rhodobacter sphaeroides*)	437-438, 621-625 (29)	(E443, F625) (17)
DVP (from cucumber)	436, 537, 573, 625 (17)	(E443, 451, F625) (22)
MVC	429, 660 (30, 10)	(E447, F675) (22)
DVC	435, 659 (30)	(E458, F675) (30)

DV-chlorophyllide by using a low temperature octadecylsilica column with an ion-pair elution method. But as pointed out by Shioi and Beale (31), this method is time-consuming and the elution system was complex compared to their technique (31). They have recently reported the separation of MV- and DV-tetrapyrrole intermediates by HPLC, using a powdered polyethylene column and a simple elution system employing aqueous acetone. The method described by Shioi and Beale (31) provides a rapid separation and also a qualitative and quantitative determination of chlorophyll intermediates present. Therefore, the method employing a polyethylene column was used for the separation of the protochlorophyllide and chlorophyllide pools, and the determination of the relative amounts of intermediates present during assays for the 4VR.

CHAPTER 2

MATERIAL AND METHODS

2.1. MATERIALS AND INSTRUMENTS.

2.1.1. *Plant material.*

Cucumber seeds (*Cucumis sativus*) were purchased from Buckerfields Co., Vancouver B.C. and wheat seedlings (Triticum aestivum) were obtained from The Department of Biological Sciences, Simon Fraser University, Burnaby, B.C..

2.1.2. *Chemicals.*

Most of the chemicals were readily available from commercial sources and were reagent grade unless otherwise specified. Sephadex G-75 and G-10 were obtained from Pharmacia Fine Chemicals, Lachine, Quebec; HPLC grade reverse phase polyethylene powder from Polyscience Inc.; Warrington, PA ; HPLC certified A.C.S.-spectroanalyzed acetone and ICN-Alumina B, Super I from ICN Biomedicals, St. Laurent, Quebec. NADPH, $NADP^+$, NADH, and ATP, were obtained either from Sigma Chemical Inc. or Boehring Manheim, Dorval, Quebec. ADH from *Thermoanaerobicum brocki* and GDH from *Bacillus sp.* were obtained from Sigma Chemical Inc.; BSA from Boehringer Manheim, Dorval, Quebec, D-[1-^{3}H] glucose (20 Ci / mol) from New England Nuclear, Mississauga, Ontario and Scintillation cocktail (Scinti-Verse II) from Fisher, Vancouver, B.C.

2.1.3. *Instruments.*

Instruments employed were the Beckman model LC 800 scintilation counter; Waters model 510 HPLC pump and HPLC column (4.6 x 250 cm); glass jacket (made in SFU glass shop); HPLC spectrophotometer - Lambda - Max model 481; Philips model PU 8 700 UV-Visible Spectrophotometer and model MP-44B fluorescence spectrophotometer .

2.2. PREPARATION OF PIGMENTS, ENZYMES, AND LABELED COENZYMES.

2.2.1. *Preparation of monovinylprotochlorophyllide from etiolated wheat seedlings.*

MVP was isolated by the method described by Griffiths (11). Wheat seedlings (50 g) were soaked in water for 1 hour with aeration and grown on moist vermiculite in the dark for 12-14 days. All of the following manipulations were performed under a dim green safelight at room temperature. Wheat seedlings were cut into small pieces and ground with mortar and pestle in 400 ml of acetone containing 400 mg $MgCO_3$. The homogenation was repeated several times until very little pigment remained in the tissue as monitored by its UV-fluorescence. The resulting homogenate was filtered through glass wool and an equal volume of water was added to the filtrate plus 10 g of NaCl. The pH of the solution was then adjusted to 6.7 with 0.1 M NaH_2PO_4. The pigment was extracted 3 times into peroxide-free ether, and the combined ether extracts were washed twice with an equal volume of distilled water. The ether extract at this stage was mixed with an equal volume of petroleum ether and the porphyrins were extracted into 20 ml of methanol / 0.01M NH_4OH (4:1). This procedure was repeated a further three times untill no more protochlorophyllide appeared in the ammoniacal methanol layer. The basic extract was then washed 3 times with 50 ml ether / petroleum ether (1:1) to remove any traces of non - polar pigments carried over into the protochlorophyllide extract. The washed extract was then adjusted to pH 6.7 by 0.1M NaH_2PO_4 and porphyrins were again extracted 3 times with 50 ml of peroxide-free ether. The combined ether extract was washed

twice with water. The ether was then evaporated under a stream of N_2, and the rest of the water was removed by freeze-drying. The dry sample was stored at -20 °C.

2.2.2. *Preparation of monovinyl -and divinylprotochlorophyllide from etiolated cucumber cotyledons.*

A mixture of MVP and DVP was isolated by the method of Belanger & Rebeiz (17). Cucumber seeds previosly soaked in water for 1 h with aeration were grown in moist vermiculite in the dark for 5-6 days. All of the following steps were performed under a dim green safelight. Etiolated hookless cucumber cotyledons (50 g) were harvested and ground with mortar and pestle in 50 ml of acetone / 0.1 M NH_4OH (9:1). Large particles were removed by decanting the solution through glass wool. The extraction was repeated until very little fluorescence remained in the etiolated cotyledons. The acetone extract was then centrifuged at 11,000 g for 10 min. The pigment was then extracted from the supernatant as described by Rebeiz *et al.* (19). The supernatant was washed twice with hexane in order to remove fully esterified tetrapyrroles and carotenoid pigments. The hexane-extracted acetone fraction was adjusted to pH 6.7 by 0.1M NaH_2PO_4 and then extracted into peroxide-free ether. In some cases, it was necessary to add a sufficient amount of saturated NaCl to break down any emulsions. The ether was evaporated under a stream of N_2 and the dry sample was stored at -20°C. The sample contained a mixture of MVP : DVP (1:1) as analysed by the HPLC method described in 2.3.2.

2.2.3. *Generation of increased amounts of divinylprotochlorophyllide in etiolated cucumber cotyledons.*

Increased amounts of DVP were generated by the method of Duggan & Rebeiz (32). Etiolated cucumber cotyledons were harvested as described in 2.2.2 under a green safelight . The generation of DVP was achieved by an irradiation of etiolated cotyledons (after harvesting) with 2.5 ms of white actinic light (L) positioned 10-15 cm above the excised tissue. The protochlorophyllide pool was regenerated by returning the tissue back to darkness for 60 min (D). This light - dark treatment was repeated two more times (3LD). Isolation and extraction of the pigments was then carried out as described in 2.2.2 and the dry sample was stored at -20°C. This sample contained a mixture of MVP and DVP (1:4) as analysed by the HPLC method described in 2.3.2.

2.2.4. *Solubilization of the protochlorophyllide pool.*

The protochlorophyllide pool was solubilized by the method of Griffiths (11) prior to its addition to enzymatic incubations by evaporating a portion of the ether stock solution to dryness under a stream of N_2 followed by addition of 0.3-0.5 ml of a methanolic - cholate solution (0.5 mg sodium cholate in 1 ml 90% aqueous methanol). The solution was evaporated to dryness under a stream of N_2 and traces of the solvent were removed by freeze-drying for 2 h or longer. This procedure gave a crystalline mixture of the pigment in sodium cholate, which was dissolved in 50 mM HEPES, pH 7.2, just prior to enzymatic incubation. In this form the pigment was stable for at least 8 h (11).

2.2.5. *Generation of the chlorophyllide pool from etiolated cucumber cotyledons.*

An attempt was made to generate DVC and MVC from the protochlorophyllide pool by the method of Belanger & Rebeiz (17). Etiolated cucumber cotyledons were either exposed to 1 light - dark (1LD) or 3 light - dark (3LD) treatments as described in 2.2.3. Immediately before extraction the cotyledons were exposed to 1 more flash (1LD+L or 3LD+L). Isolation, extraction and storage of the resulting pigments was caried out as described in 2.2.2. Additional, MVC, either produced by the action of chlorophyllase on chlorophyll *a* isolated and purified from spinach or extracted from etiolated oat seedlings after light treatments, were donated by J.S. Wieler (33) and by P. Lauterbach (34), respectively.

2.2.6. *Preparation of wheat etioplasts and lysed wheat etioplast membranes.*

Wheat etioplasts were prepared by the method of Griffiths (35). Wheat seedlings were grown for 10 days in the dark, and harvested as described in 2.2.1.The temperature during the whole procedure was maintained betwen 0-4^{o}C. Cut wheat seedlings (120 g) were blended for 1 min in an Ato - Mix blender in Buffer I (pH = 7.2) composed of 0.5 M sucrose, 0.2% (w / v) BSA, 5 mM cysteine, 1mM $MgCl_2$, 1mM EDTA, 20 mM TES, and 10 mM HEPES and adjusted with saturated KOH to pH 7.2. The homogenate was filtered through four layers of cheesecloth and four layers of muslim and centrifuged at 9,000 g. The centrifuge was rapidly stopped after reaching 9,000 g and the resulting pellet was resuspended in Buffer I. The solution was then centrifuged at 1,500 g for 90 s in order to remove cellular debris. The etioplasts were sedimented from the resulting supernantant by a further centrifugation at 6,000 g for 90 s. The etioplast pellet was finally resuspended in 20 ml of Buffer I enriched by 1.5 mM ATP.

etioplast pellet was finally resuspended in 20 ml of Buffer I enriched by 1.5 mM ATP.

Lysed etioplast membranes were prepared from the etioplast pellet osmotically lysed by the method of Beer & Griffiths (36). Five ml of the resuspended etioplast pellet was centrifuged to 8,000 g and the motor was immediately switched off after reaching this point. The resulting pellet was resuspended in 5 ml of Buffer II (pH 7.5) containing 20 mM sucrose, 20 mM TES, 20 mM HEPES and 2 mM $MgCl_2$, which caused lysis of the etioplasts. The etioplast membranes were sedimented by centrifugation at 91,000 g for 20 min and then resuspended in 8 ml of Buffer III (pH 7.0) containing 5 mM Tricine, 20 mM HEPES, 2 mM $MgSO_4$, 1 mM EDTA and 4 mM CHAPS in 25% aqueous glycerol (37).

2.2.7. *Preparation of cucumber etioplasts and lysed cucumber etioplast membranes.*

Cucumber etioplasts were prepared according to the method of Duggan & Rebeiz (8). Cucumber seeds were germinated and cotyledons prepared as described in 2.2.2. The etiolated cucumber cotyledons were exposed to three light -dark treatments as described in 2.2.3 in order to accumulate more DVP (8, 32,). Five g batches of these cotyledons were homogenized in 12.5 ml of Buffer IV (pH 8) containing 0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 30 mM TES, 15 mM HEPES, 5 mM cysteine, and 2% (w / v) BSA (40) by the use of a mortar and pestle. The homogenate was passed through four layers of cheesecloth and the resulting homogenate was centrifuged at 200 g for 3 min in order to remove heavy particles. The supernantant was then centrifuged for 7 min at 1,500 g. The pelleted etioplasts were resuspended in Buffer V (pH 7.7) containing of 0.5 M

sucrose, 0.2 M Tris - HCl, 20 mM ATP, 1 mM NADP, 1.25 mM methanol and 1% (w / v) BSA .

Lysed etioplast membranes were prepared by the method of Richter and Rienitis (38) by resuspending the final etioplast pellet in a hypotonic lysing medium (Buffer VI) which contained 20 mM TES, 10 mM HEPES, 5 mM GSH, 5 mM $MgCl_2$, 1mM EDTA, 10 mM KH_2PO_4, 0.6 mM NADP and 5 mM ATP adjusted to pH 7.7 with saturated KOH. The homogenate was centrifuged at 600 g for 90 s. The resulting supernantant was then centrifuged at 23,000 g for 15 min. The pellet of lysed etioplast membranes was resuspended in fresh Buffer VI.

The etioplasts and lysed etioplast membrane were both used fresh immediately after their preparation.

2.2.8. *Extraction of the pigments from etioplasts and lysed etioplast membranes.*

All of the reactions described in chapter 2.4. and 2.5. were stopped by the addition of acetone /0.1 M NH_4OH (9:1) and centrifuged at 39,000 g for 10 min. The ammoniacal acetone fractions were adjusted to the same volume by the addition of 75% aqueous acetone, and extracted with an equal volume of hexane followed by a 1/ 3 volume of hexane. Following the addition of 1/17 of its volume of saturated NaCl and 1/70 of its volume of 0.5 M KH_2PO_4, the hexane-extracted acetone was then extracted with an equal volume of peroxide free-ether, followed by 2 more extractions with half that volume of ether. The combined ether extracts were concentrated under a stream of N_2 before analysis.

2.2.9. *Preparation of stereospecifically tritium labeled coenzymes : the 4R- and 4S- isomers of [^{3}H]NADPH.*

The stereospecifically tritium labeled 4S- and 4R- isomers of [^{3}H] NADPH were prepared by the method of Richards *et al.* (39) (Fig 2.2.1). GDH from *Bacillus sp.* which is specific for the pro S hydrogen of either NADH or NADPH (40), was used to transfer a tritium atom from [1-^{3}H] glucose to the β face of $NADP^+$ to give [4S-^{3}H]NADPH. This incubation was followed by a second incubation with acetone and ADH from *Thermoanaerobium brockii,* which is specific for the pro R hydrogen of NADPH (40), yielding [4-^{3}H]$NADP^+$. The [4-^{3}H]$NADP^+$ was then incubated with GDH which transfered a hydrogen atom from an unlabeled glucose to give [4R-^{3}H]NADPH.

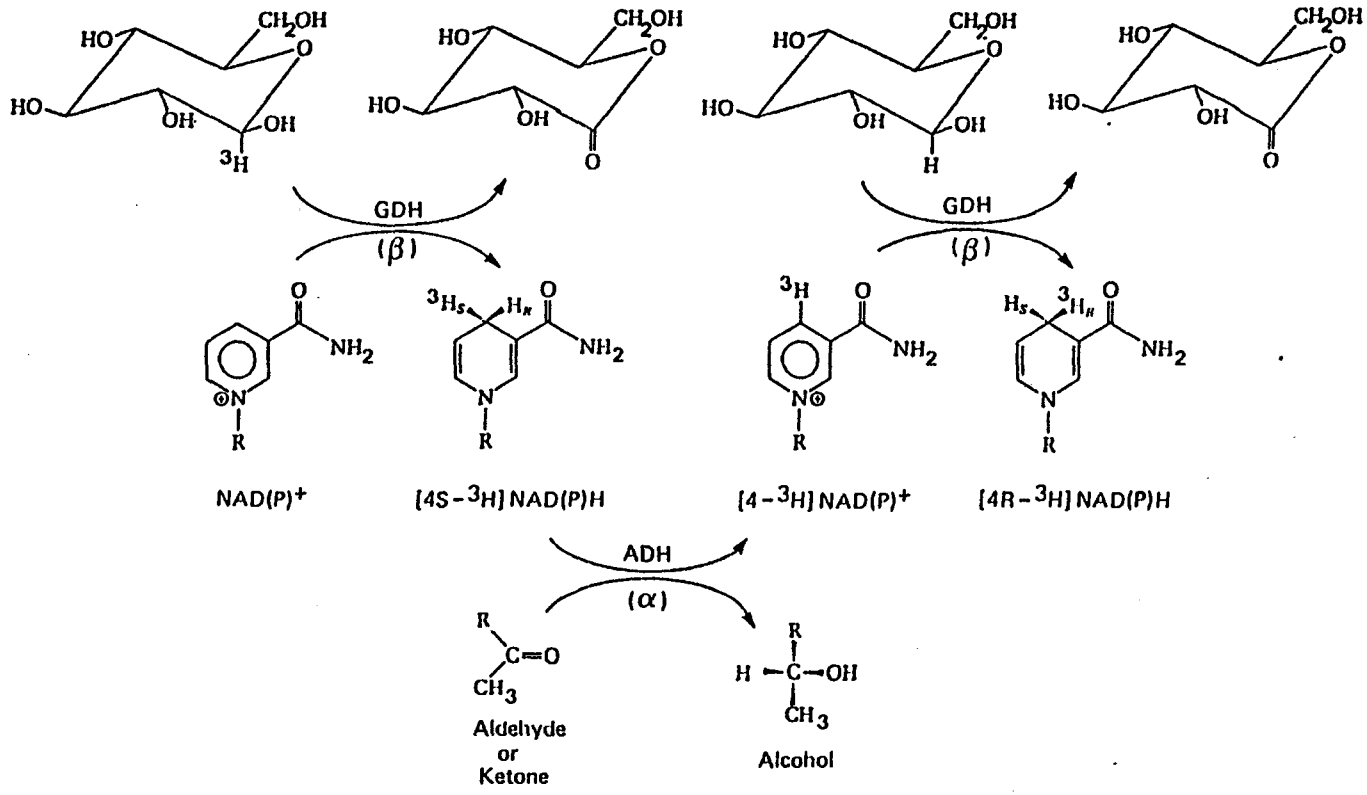
2.2.9.1. *Preparation of tritium labeled [4-^{3}H]$NADP^+$.*

[4-^{3}H]$NADP^+$ was synthetized by incubation of 10 ml 0.5 mM $NADP^+$ and D-[1-^{3}H] glucose (2 Ci mol^{-1}, New England Nuclear) and 10 units of GDH from *Bacillus sp.* in a phosphate buffer (0.02 M K_2HPO_4, pH 7.2) for 90 min at room temperature. The reaction went 60% to completion on the base of A_{340}. This incubation was followed by a second incubation with 10 mM acetone and 10 units of ADH from *Thermoanaerobicum brockii* for 15-20 min at room temperature. Based on A_{340} the reaction went 100% to completion. The final product, [4-^{3}H] $NADP^+$, was purified by Sephadex G-75 in a 2 x 40 cm glass column. The flow rate of the above phosphate buffer was adjusted to 0.68 ml min^{-1}. A total of 35 fractions with a volume of 3.7 ml each were collected and analysed for radioactivity by counting 10 µl aliquots of each fraction in 10 ml of liquid scintillation cocktail . The [4-^{3}H] $NADP^+$ was eluted betwen 22.9 ml to 55.5

Fig 2.2.1

The scheme for the preparation of stereospecifically (4S and 4R) tritium labeled NAD(P)H. The enzymes, glucose dehydrogenase (GDH) and alcohol dehydrogenase (ADH), are specific for the pro S (or β) and pro R (or α) hydrogens of NAD(P)H.

GENERATION OF STEREOSPECIFICALLY LABELED NAD(P)H



ml (Fig 2.2.2). The fractions collected between 30-51 ml were concentrated by freeze drying . Further purification of the concentrated fraction was carried out on Sephadex G-10 in a 2 x 40 cm glass column. The flow rate of elution buffer was adjusted to 0.75 ml min^{-1} and 35 fractions (of 3.7 ml each) were collected and analysed for radioactivity by counting 10 µl of each fraction in 10 ml of liquid scintillation cocktail. The 7 fractions with the highest activity (14.8 ml-40.7 ml) were also analysed for A_{260}, confirming that all 7 fraction contained $NADP^+$. Labeled $NADP^+$ was eluted near to the void volume and labeled unreacted glucose (38) was eluted near the bed volume (40.7-55ml) (Fig. 2.2.3) . Any labeled isopropanol produced by continued enzymatic cycling would have been removed during the freeze drying of the sample betwen the two gel filtrations. Quantitative analysis for tritium-labeled $NADP^+$ was done after the fractions were pooled. The amount of purified [4-^{3}H] $NADP^+$ was calculated on the basis of its A_{260} and 3.5 µmol were recovered. An aliquot of 50 µl of the sample was counted in 10 ml of liquid scintillation cocktail. The results indicated that in the [4-^{3}H] $NADP^+$ solution contained 3.68 µCi and its final specific activity was found to be 1.04 Ci mol^{-1}.

2.2.9.2. *Generation of stereospecificaly labeled [4R-^{3}H] and [4S-^{3}H] NADPH.*

Stereospecifically reduced nicotinamide coenzymes were generated just prior to the experiment as follows:

1) Generation of [4R-^{3}H]NADPH

A total of 1 µmol of [4- ^{3}H] $NADP^+$ (1 Ci mol^{-1}) was incubated for 40 min at room temperature (21-22°C) in 1 ml of 0.02 M KH_2PO_4 (pH 7.2) with 2 µmol of unlabeled glucose, and 1 unit of GDH. This incubation yielded [4R-^{3}H]NADPH

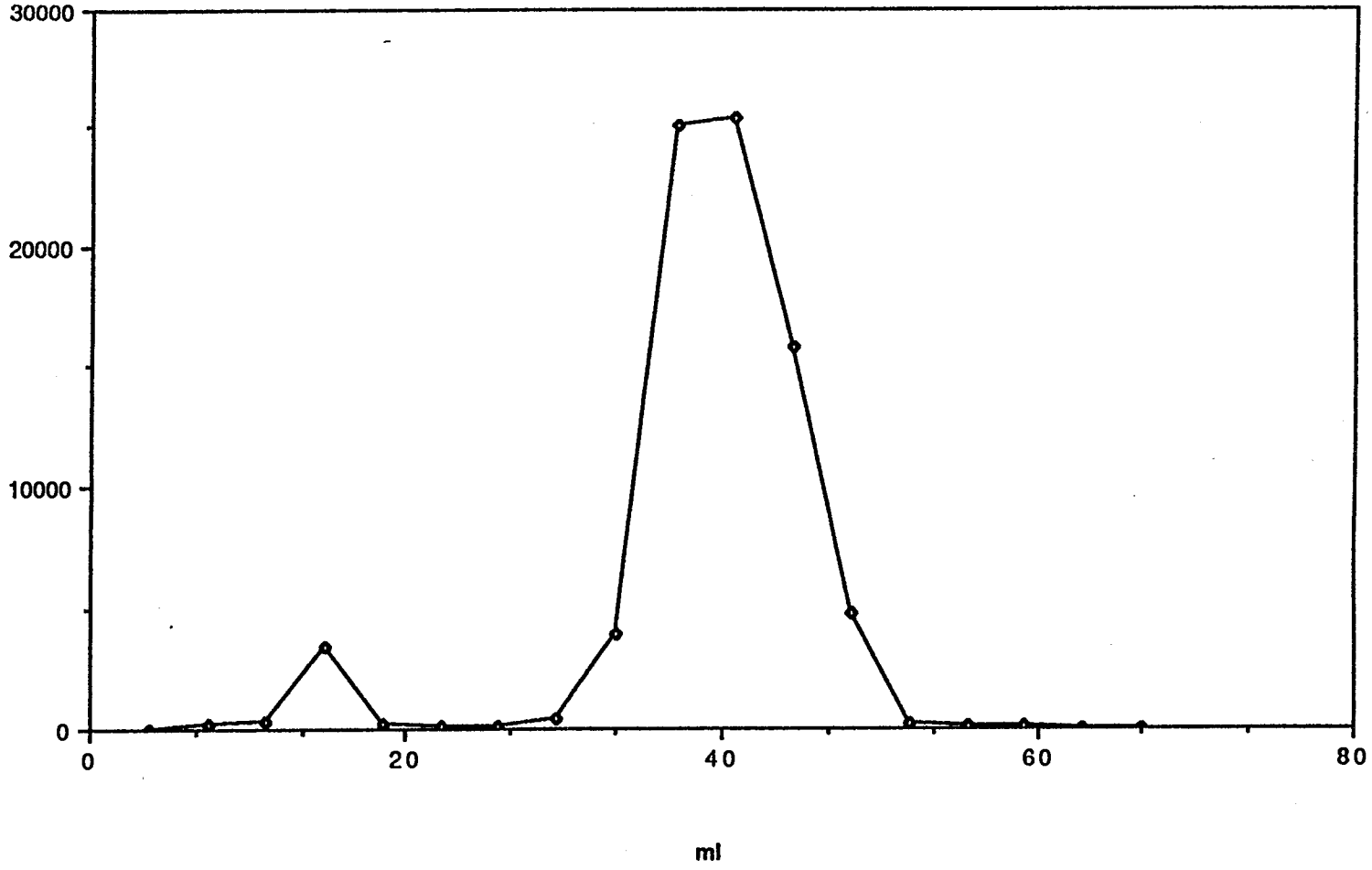
and on the basis of its A_{340}, the conversion of [4-^{3}H] $NADP^+$ to [4R-^{3}H] NADPH was 69%.

2) Generation of [4S-^{3}H]NADPH.

A total of 1 μmol of $NADP^+$ was incubated for 40 min at room temperature in 1 ml of 0.02 M KH_2PO_4 (pH 7.2) with 2 μmol D-[1-^{3}H] glucose (at a final specific activity of 1Ci mol^{-1}), and 1 unit GDH, yielding [4S-^{3}H]NADPH. On the basis of its A_{340}, the conversion of $NADP^+$ to [4S-^{3}H]NADPH was 85%.

Fig 2.2.2

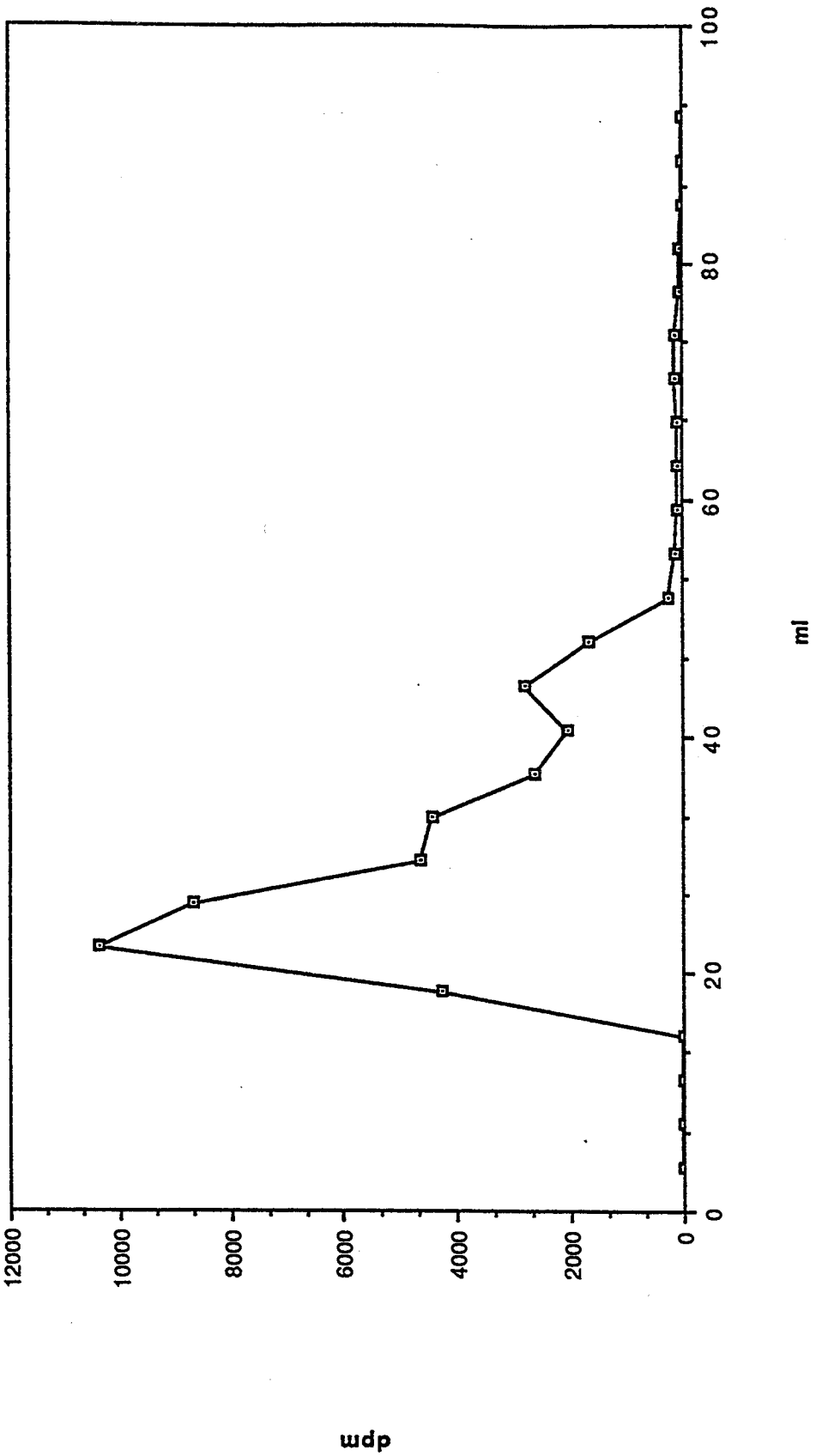
Gel filtration on Sephadex G-75 of [4-^{3}H]NADP^{+} prepared as described in Chapter 2.2.9.1. Fractions were analyzed for radioactivity only.



20b

Fig 2.2.3

Gel filtration on Sephadex G-10 of [4-^{3}H]NADP^{+} prepared as described in Chapter 2.2.9.1. Fractions were analyzed for radioactivity only.


12000
10000
8000
6000
4000
2000
0
dpm
0
20
40
60
80
100
ml

2.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

2.3.1. *HPLC separation of the protochlorophyllide pool by the use of a powdered polyethylene column.*

The separation of the protochlorophyllide pool by HPLC was performed according to the method of Shioi and Beale (31). The HPLC column was packed with reverse phase HPLC grade polyethylene powder with the aid of a plastic stick. To compress the packing material, the column was run at a pressure of 160-190 kg cm^{-2}. This process was repeated several times until no void remained at the top of the column. The presence of a void volume results in a decrease in column efficiency. According to Shioi and Beale (31), resolution of the pigment can be controlled by varying the mobile phase polarity and the temperature of the run. They selected, as a mobile phase for isocratic separations, 65% aqueous acetone at a flow rate of 0.2 ml min^{-1} at 20°C. The pigment was dissolved prior to injection in 65% aqueous acetone. .

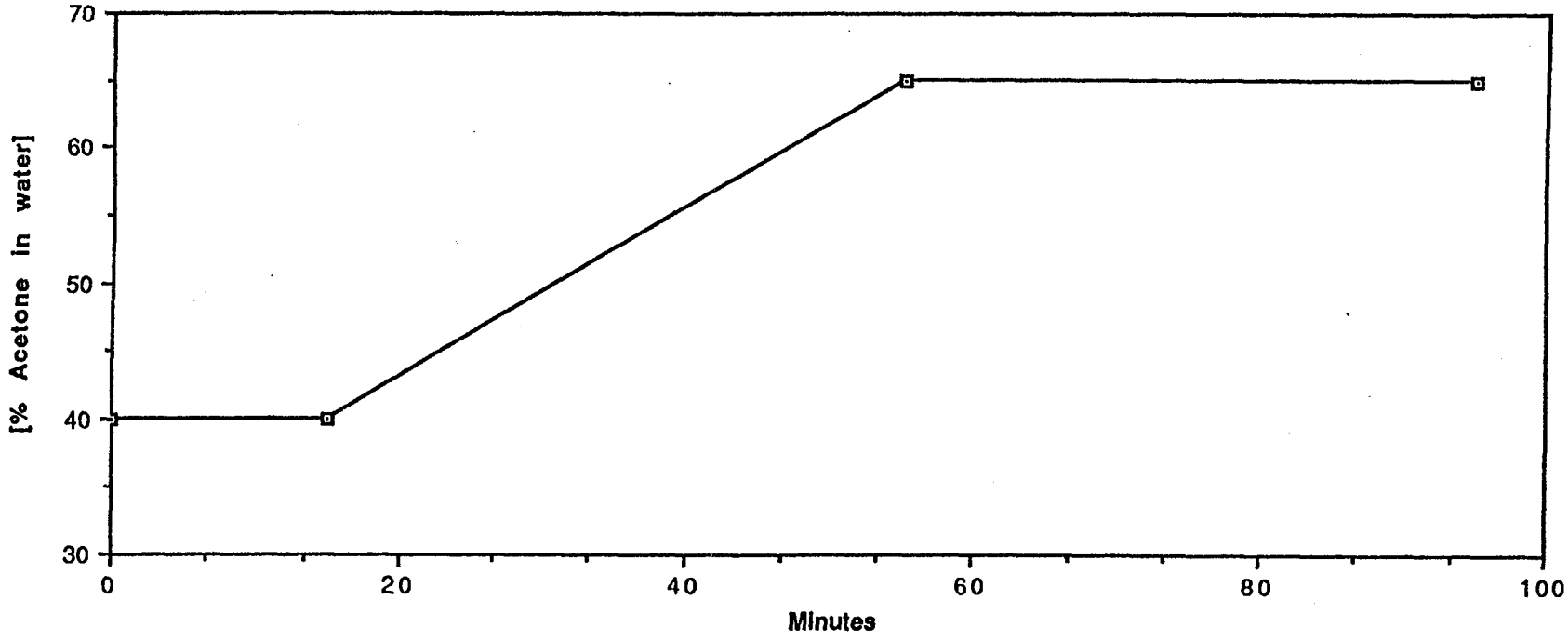
2.3.2. *A new HPLC method for the separation of monovinyl- and divinylprotochlorophyllides and chlorophyllides.*

Conditions for the separation of the protochlorophyllide and chlorophyllide pools on a single run were established by using a new HPLC solvent system. The column was packed with reverse phase HPLC grade polyethylene powder as described in 2.3.1. The column was encased in a glass water jacket and the flow rate was adjusted to 0.2 ml min^{-1}, the temperature of the water bath was 20°C. The solvent system was established as follows: The column was washed overnight with 40% aqueous acetone. Following injection of the sample in 40%

aqueous acetone, the method starts with an isocratic run of 40% aqueous acetone for 15 min, followed by a gradient run of from 40% to 65% aqueous acetone for 40 min and finishing with an isocratic run of 65% aqueous acetone for 40 min. The method is shown in Fig. 2.3.1.

Fig 2.3.1

The scheme of the new isocratic-gradient-isocratic HPLC solvent system for the separation of the protochlorophyllide and chlorophyllide pools on a single run by powdered polyethylene column. The method is described in Chapter 2.3.2.


70
60
50
40
30
[% Acetone in water]
0
20
40
60
80
100
Minutes

2.4. ASSAY FOR THE WHEAT ENZYMES.

TABLE 3.4.1 summarizes experiments in which 4VR and PCR enzyme activities were observed in wheat etioplasts and lysed etioplast membranes. Exogenous pigment (80% DVP and 20% MVP) was prepared and solubilized by the cholate procedure as described in 2.2.4. The compositions of Buffers I, II, and III are given in section 2.2.6. All steps were performed under a dim green safelight, and the reactions were stopped by the addition of 5 ml of acetone / 0.1 M NH_4OH (9:1). The pigments were extracted as described in 2.2.8.

2.4.1. Assay for wheat enzymes using whole etioplasts.

Experiment **A.**

To 5 ml of wheat etioplasts resuspended as described in 2.2.6, 1 ml of 50 mM HEPES containing 3.66 µmol of the exogenous pigment described above and 1 ml of 7.5 mM NADPH was added. Samples were preincubated in a shaking water bath at 25°C in the dark for 3 min. All samples were then irradiated with 4 actinic light pulses with 20 s dark intervals between them. After a fifth and final light pulse (5L), the samples were incubated in a shaking water bath at 25°C in the dark for various lengths of time before stopping the reaction.

2.4.2. Assay for wheat enzymes using lysed etioplast membranes.

Experiment **B.**

Lysed etioplast membranes were prepared from 2 ml of resuspended etioplasts as described in 2.2.6. The resulting membrane pellet of the lysis procedure was resuspended in 8 ml of Buffer III and adjusted to 10 ml before

adding 0.5 ml of 7.5 mM NADPH. To 2 ml volumes of each sample of this solution was added 0.2 ml of 50 mM HEPES containing 5.25 nmol of exogenous pigment. Dark incubation was carried out immediately after adding pigment in a shaking water bath at 25°C.

Experiment **C.**

Lysed etioplast membranes were prepared from 2 ml of resuspended etioplasts as described in 2.2.6. The resulting membrane pellet was resuspended in 6 ml of Buffer III and adjusted to 8 ml. To this volume was added 1 ml of 7.5 mM NADPH and 0.3 ml of 50 mM HEPES containing 0.65 nmol of exogenous pigment. Samples were incubated in the dark for various lengths of time at 4°C.

Experiment **D.**

Lysed etioplast membranes were prepared from 1 ml of resuspended etioplasts as described in 2.2.6.. The resulting membrane pellet was resuspended in 6 ml of Buffer III. To each of 3 ml of this homogenate was added 0.1 ml of 50 mM HEPES containing 0.22 nmol of pigment. Samples were irradiated with 22 light pulses with 10 s dark intervals (22 L). The samples were incubated in the dark for 0 s and 20 min at 25°C.

Experiment **E.**

The lysed etioplast membranes were prepared from 1 ml of resuspended etioplasts as described in 2.2.6.. The resulting membrane pellet was resuspended in 8 ml of Buffer III to which was added 0.5 ml of 7.5 mM NADPH and 1 ml of 50 mM HEPES containing 2 nmol of pigment. Samples, of 3 ml volume each, were exposed to 16 light flashes with 10 s dark intervals, followed by 3 fast light flashes (16L + 3) before dark incubation at 26°C for various lengths of time.

2.5. ASSAY FOR THE CUCUMBER ENZYMES.

TABLE 3.5.1 summarizes experiments in which the 4VR and PCR enzyme activities were observed in cucumber etioplasts and lysed etioplast membranes using endogenous pigments. In the experiments described below, the reactions were stopped by the addition of 10 ml of acetone / 0.1N NH_4OH (9:1). All samples were centrifuged at 39 000 g for 10 min and the pigments were extracted into peroxide-free ether as described in 2.2.8.

2.5.1. *Assay for cucumber enzymes using whole etioplasts.*

Cucumber etioplasts were prepared from 30 g (A) or 45 g (B) of etiolated cucumber cotyledons as described in 2.2.7. The resulting pellets were resuspended in 21 ml (A) or 25 ml (B) of Buffer V. Samples were preincubated in a shaking water bath (50 osc. min^{-1}) for 3 min at 25°C.

In **A**, three 7 ml samples of this homogenate were diluted with 3 ml of distilled water. Sample **a** was left in the dark, while samples **b** and **c** were exposed to one 2.5 ms actinic light pulse (1L) and incubated for various lengths of time as given in **Table 3.5.1.**

In **B**, five 5 ml samples of this homogenate were diluted with 2 ml of distilled water. Sample **d** was left in the dark while samples **e, f, g,** and **h** were exposed to two 2.5 ms actinic light flashes with 20 s of dark intervals, followed by one more light pulse(3L) before incubation for various lengths of time as given in **Table 3.5.1.**

2.5.2. *Assay for cucumber enzymes using lysed etioplast membranes.*

Lysed etioplast membranes were prepared from 35 g of 5 day - old etiolated cucumber cotyledons as described in 2.2.7. The resulting pellet was resuspended in 10 ml of Buffer VI and adjusted to 12 ml. To this homogenate was added 0.5 ml of a solution containing 7.5 mM NADPH and 7.5 mM NADH. Six 2 ml samples were diluted by 1 ml of distilled water and preincubated in a water bath at 27^{o}C for 3 min.Samples **i** and **j** were left in the dark while samples **k, l, m** and **n** were exposed to two 2.5 ms light pulses with 20 s dark intervals followed by a final light flash (3L). Incubation was carried out in the dark at 27^{o}C for various lengths of time as shown in **Table 3.5.1.**

2.5.3. *Assay for cucumber enzymes using lysed etioplast membranes in the presence of the 4R- or 4S-isomer of [^{3}H]NADPH.*

Lysed cucumber etioplast membranes were prepared from 60 g of 5 day-old etiolated cotyledons as described in 2.2.7, except that the etiolated cucumber cotyledons were not previously exposed to 3 light-dark treatments. The resulting membrane pellet was resuspended in 12 ml of Buffer VI. Six 1.7 ml samples were diluted with 2 ml of 0.02 M KH_2PO_4 (pH 7.2) and preincubated in a shaking water bath (50 osc. min^{-1}) at 27^{o}C for 3 min. Sample **a** was left in the dark, while samples **b, c, d** and **e** were exposed to 5 light pulses with 20 s dark intervals betwen them, followed by a final light pulse (6L). After the final light pulse, the samples were treated as follows: The incubation of sample **b** was immediately stopped after the last light pulse. To samples **d, e** and **f** were immediately added 2 ml of 0.02 M KH_2PO_4 (pH 7.2) containing either 1μmol of NADH (d), 1μmol of [4S-^{3}H]NADPH (specific activity 1Ci mol^{-1}) (e), or 1μmol of [4R-^{3}H]NADPH (specific activity1Ci mol^{-1}) (f).

Samples **a, c, d, e** and **f,** were incubated in the dark at 27°C for 10 min before stopping the reaction. **Table 3.7.1** summarizes the various treatments for each of the samples.

All samples were analysed by the HPLC method described in Chapter 2.3.2 In the case of samples **e** and **f,** 40 fractions from the HPLC column were collected (each containing 0.5 ml). and 100 µl of each was bleached with 20 µl of 3% hydrogen peroxide and counted in 10 ml of liquid scintilation cocktail.

CHAPTER 3

RESULTS

3.1. THE ABSORPTION AND FLUORESCENCE SPECTRA OF THE PROTOCHLOROPHYLLIDE AND CHLOROPHYLLIDE POOLS.

The absorption and low temperature fluorescence spectra of MVP isolated from etiolated wheat seedlings are nearly identical to the published spectra (11, 19). The absorption spectra (Fig. 3.1.1) of 1:1 and 1:4 mixtures MVP and DVP isolated from etiolated cucumber cotyledons gave maxima at 434, 536, 574, 625 nm and 438, 537, 576, 626, 664 nm, respectively, and were close to the published values (32). The peak at 664 nm, which appeared after the light-dark treatments, may represent MVC, which would have remained in the ether fraction while chlorophyll would have been extracted into the hexane fraction. The fluorescence maxima of both mixtures are also close to published values (17, 19, 22) (Table 1.2.1) and are shown in Fig. 3.1.2 and Fig. 3.1.3. The slight differences in wavelength in the unpurified samples when compared to the published values are probably due to the interference of impurities present in the samples.

Several attempts to generate DVC as a possible substrate for the 4VR in etiolated cucumber cotyledons were made by the method described in Chapter 2.2.4. Samples were assayed after a variety of different light treatments of etiolated cucumber cotyledons. But the fluorescence spectra in all cases exhibited an excitation maximum at (E447,F675), which suggested that only MVC had accumulated by this treatment. Therefore, in the experiments described in chapter 3.4-3.7 we tried to generate DVC *in situ* as proposed first by Duggan & Rebeiz (8).

Fig. 3.1.1.

Absorption spectrum in ether of MVP : DVP (1:1) (solid line) and MVP : DVP (1:4) (broken line) generated in etiolated cucumber cotyledons after 3 sequential cycles of actinic light followed by 60 min darkness (3LD) .

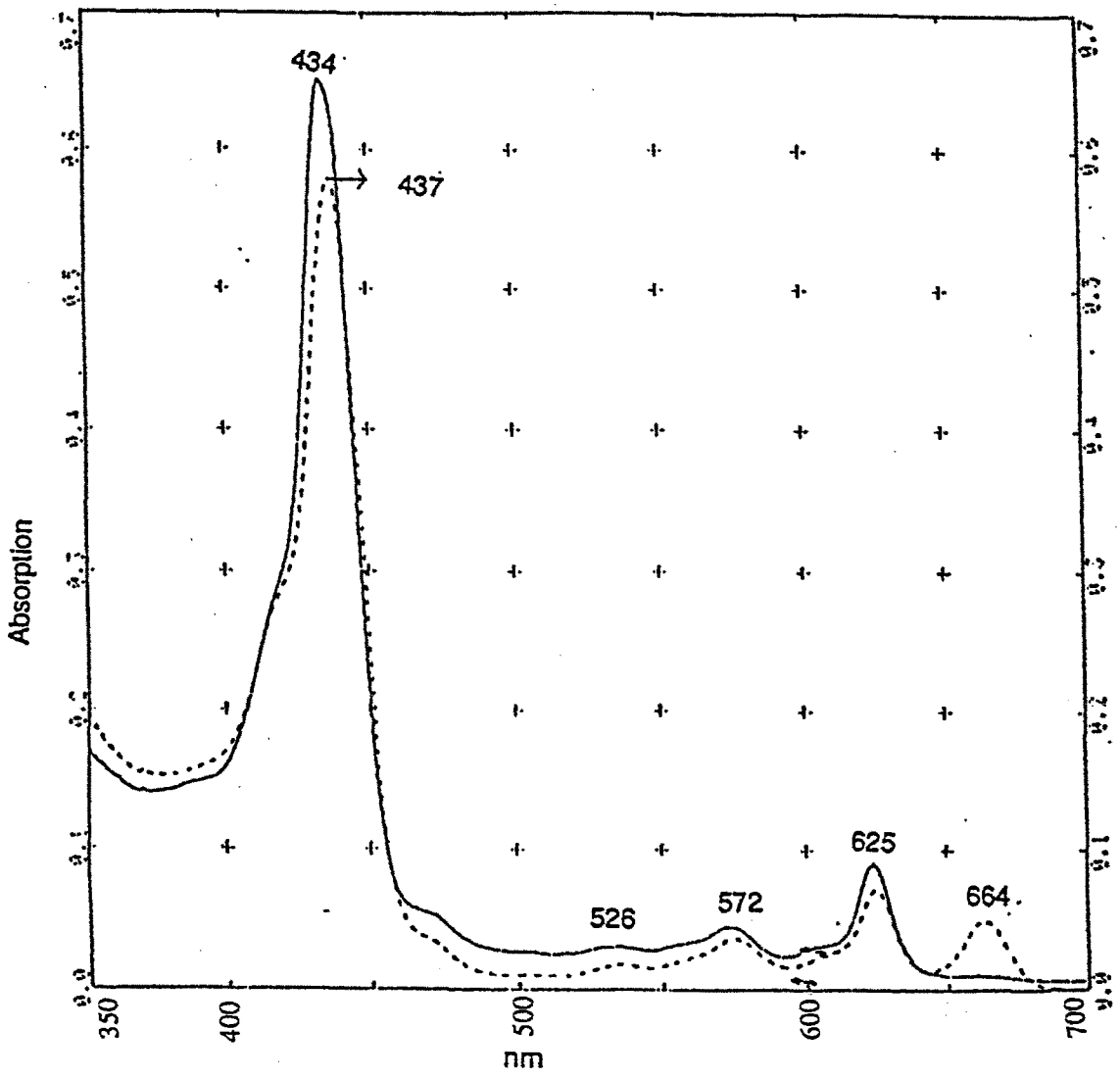

434
437
526
572
625
664
Absorption
nm
350
400
500
600
700

Fig. 3.1.2.

Fluorescence excitation spectra of MVP isolated from etiolated wheat seedlings (a), MVP and DVP (1:1) isolated from etiolated cucumber cotyledons (b), and MVP : DVP (1:4) isolated from etiolated cucumber cotyledons after 3 sequential cycles of actinic light, followed by 60 min of darkness (3LD); in ether at 77^{o}K, with emission monitored at 625 nm.

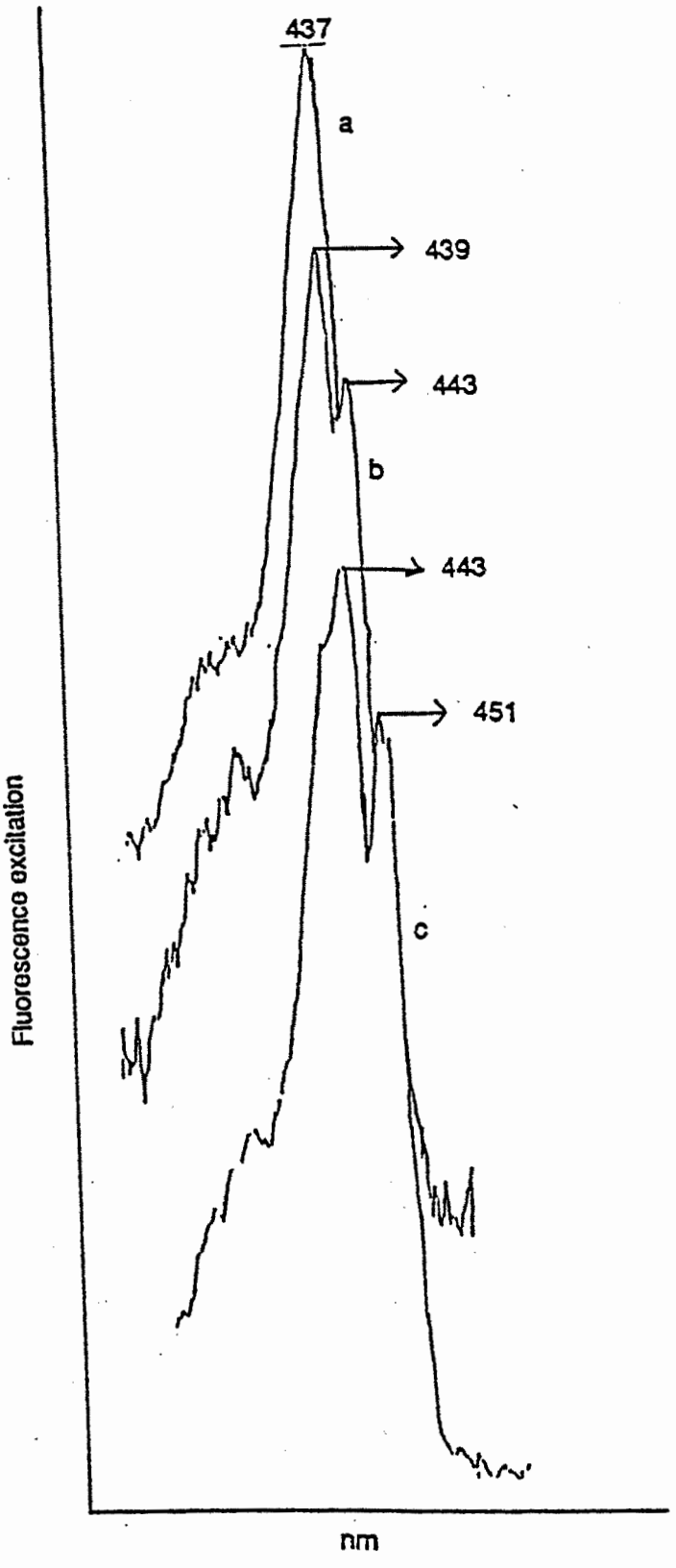
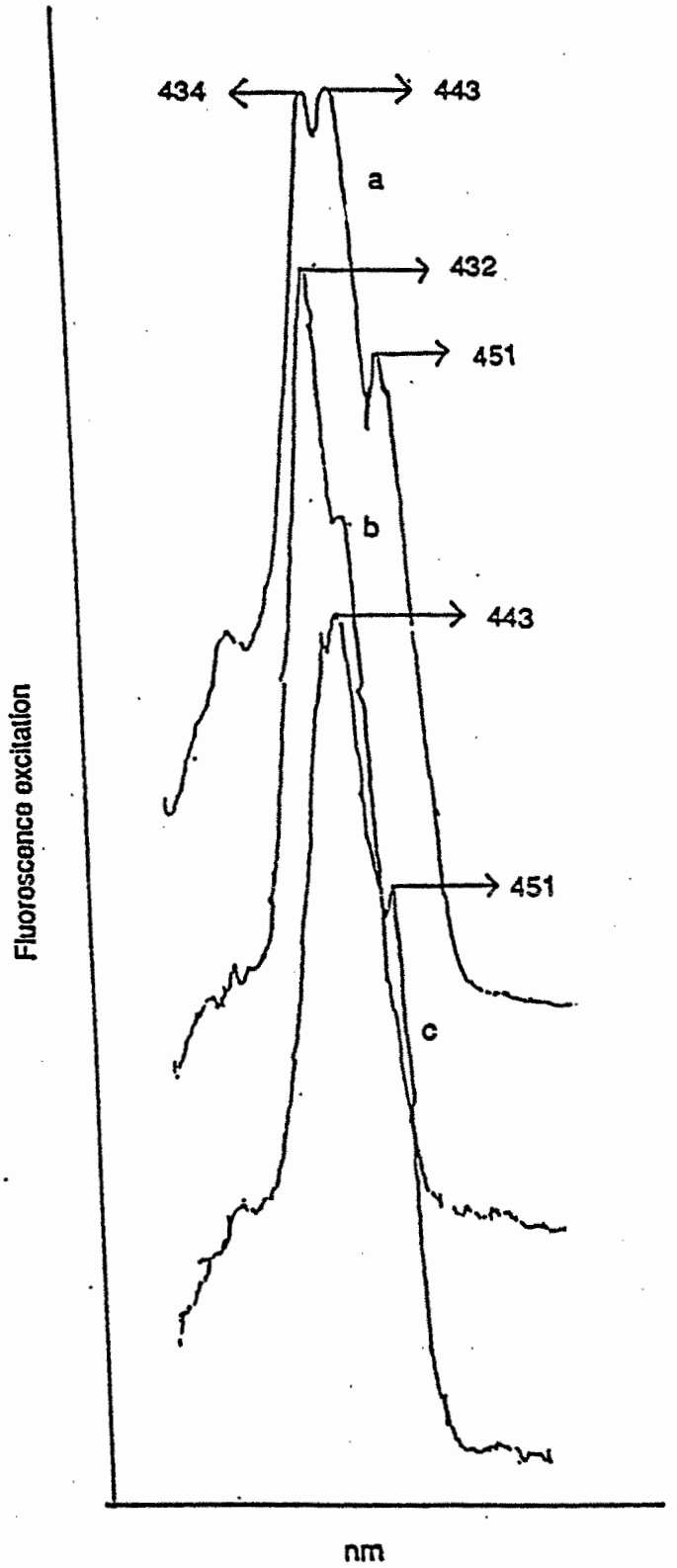

437
a
439
443
b
443
451
c
Fluorescence excitation
nm

Fig. 3.1.3.

Fluorescence excitation of MVP:DVP (1:1) isolated from etiolated cucumber cotyledons (a) and MVP (b) and DVP (c) partially purified on a polyethylene column recorded in ether at 77^{o}K with emission monitored at 625 nm.


434
443
a
432
451
b
443
451
c
Fluorescence excitation
nm

3.2. INITIAL EXPERIMENTS ON THE HPLC SEPARATION OF CHLOROPHYLL INTERMEDIATES.

The first requirement for an analysis of monovinyl- and divinyl- intermediates in chlorophyll synthesis was to find a reliable assay for the 4VR enzyme. One of the possibilities for the separation and purification of protochlorophyllide and chlorophyllide pools, the HPLC method of Shioi and Beale (31), was investigated. A mixture of MVP and DVP (prepared, respectively, from etiolated cucumber cotyledons and etiolated wheat seedlings) was run isocratically in 65% and 55% aqueous acetone according the method of Shioi and Beale (31). However, we were unable to achieve reproducible results by this method. One of the reasons for this failure was considered to be contamination of the isolated samples. Our results and those of Belanger & Rebeiz (17), indicated that the protochlorophyllide pool of etiolated cucumber cotyledons is made up of MVP and DVP and two additional fluorescent compounds of unknown identity, which are referred to as a compound (E453,F640) and compound (E451,453,F625). Therefore, samples were partially purified by running them through a polyethylene column (22 x 2.5 cm) in 65% aqueous acetone. However, even after this partial purification we were not able to achieve a reproducible separation of the protochlorophyllide pool by the published HPLC method (31).

Due to the low reproducibility of this method, it was necessary to develop new conditions and solvent systems for the separation of the MV- and DV-protochlorophyllide and chlorophyllide pools, as described in the following section.

3.3. A NEW HPLC SOLVENT SYSTEM FOR THE SEPARATION OF CHLOROPHYLL INTERMEDIATES.

Another reason for the low reproducibility of the method of Shioi & Beale (31) method described above was attributed to variable physical properties of the polyethylene powder. As pointed out by Shioi & Beale, the resolution of the protochlorophyllide pool increased with increasing polarity of the mobile phase and decreasing column temperatures. Our results suggested also, that washing the column before the injection with a more polar solvent increased the resolution of the pigments. Therefore, it was decided to establish new HPLC conditions as follows:

1) Wash the column with 40% aqueous acetone overnight before the run. The same solvent is then used for solubilization of the injected pigment in order to absorb the sample onto the column and to avoid flushing it immediately through the column.

2) Separate all four tetrapyrroles (MV- and DV- protochlorophyllide and chlorophyllide) during a single run by the use of a new isocratic - gradient - isocratic system developed as described in Chapter 2.3.

Table 3.3.1 gives the retention times and relative proportions calculated from the integrated areas of absorption at 436 nm during the HPLC separation of pigments from samples A - E as shown in Fig 3.3.1 - 3.3.3. HPLC peaks were identified by comparison of their retention times with those obtained from authentic samples. In case A (Fig 3.3.1), the peaks corresponding to MV- and DV- protochlorophyllide and chlorophyllide were isolated and each of them analysed by low temperature fluorescence spectroscopy in ether (see Chapter 3.6).

Samples were dissolved prior to injection in 40% aqueos acetone. The cloudiness in this solution is sometimes caused by an unknown compound remaining in the sample which is insoluble in acetone and soluble in water. Even if the sample remains cloudy, it is still possible to inject it, which leads to elution of a peak described by Shioi & Beale (31) as an unknown degradation pigment, with a retention time betwen 5-12 min.

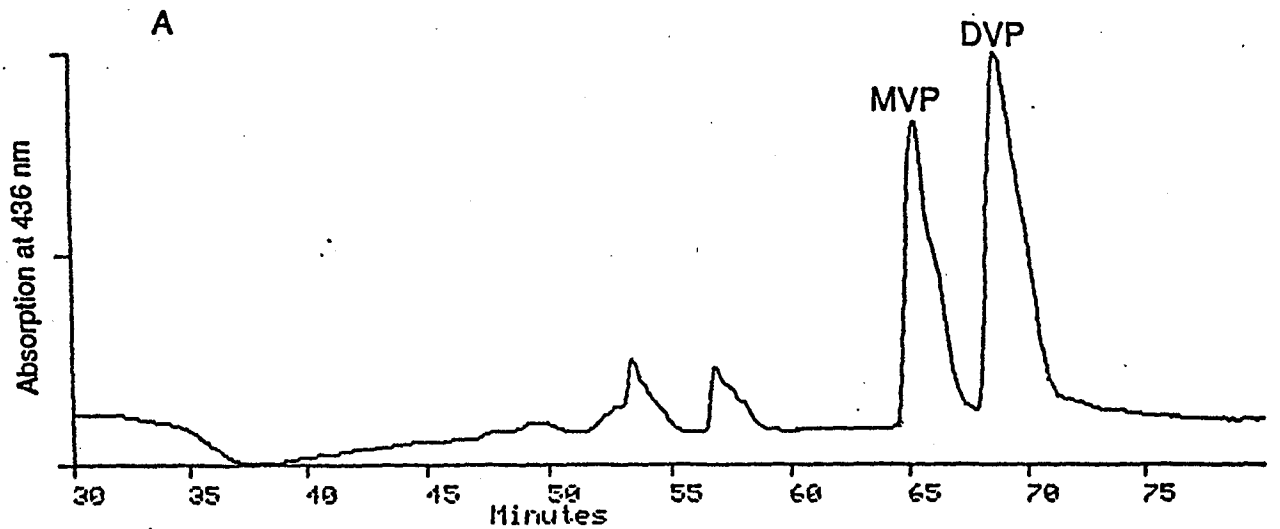
In order to obtain reproducible retention times of the separated pigments, it is important to equilibrate polyethylene columns with solvent prior to injection. The column pressure is increased by running a solvent with higher content of water. If the pressure is lower than what is normally observed for 40% aqueous acetone, it is likely that solvent with a higher content of acetone is still absorbed onto the column. This causes early elution and poor resolution (or no separation at all) of the pigments. On the other hand, if the pressure of the column is higher than what is normally observed for 40% aqueous acetone, a later retention time of separated pigment is caused. From our observations we noticed that the retention times of the pigments in the protochlorophyllide pool are more sensitive to these conditions than those of the pigments in the chlorophyllide pool. On the basis of our results the new HPLC solvent system described above seemed to be the most reliable assay for MV - and DV - protochlorophyllide and chlorophyllide and, therefore, for studying the properties of the 4VR enzyme.

TABLE 3.3.1 Summary of the retention times and relative proportion of the separated pigments from different samples shown in Figs. 3.3.1. - 3.3.3..

sample isolated from:	MVP [%]	DVP [%]	Ret. time of MVP [min]	Ret. time of DVP [min]	Ret. time of MVC [min]	Ret. time of DVC [min]	Figure
A cucumber in dark (see 2.2.2.)	42	58	66	69	/	/	3.3.1.
B cucumber 3LD (see 2.2.3.)	23	77	65.5	69.1	/	/	3.3.1.
C oat (MVC) (see 2.2.5.)	/	/	/	/	47.7	/	3.3.2.
D spinach (MVC) (see 2.2.5.)	/	/	/	/	47.5	/	3.3.2
E cucumber (mixed **g** and **m** from Tab. 3.5.1.)			69.7	74.7	49.9	52.5	3.3.3.

Fig. 3.3.1.

HPLC elution profile of MVP:DVP (1:1) (**A**) isolated from etiolated cucumber cotyledons (see 2.2.2.) and MVP:DVP (1:4) (**B**) after 3 sequential light pulses with 60 min intervening dark periods (see 2.2.3.). Samples were run by the HPLC method described in 2.3.2. and monitored at 436 nm.


A
DVP
MVP
Absorption at 436 nm
30
35
40
45
50
55
60
65
70
75
Minutes

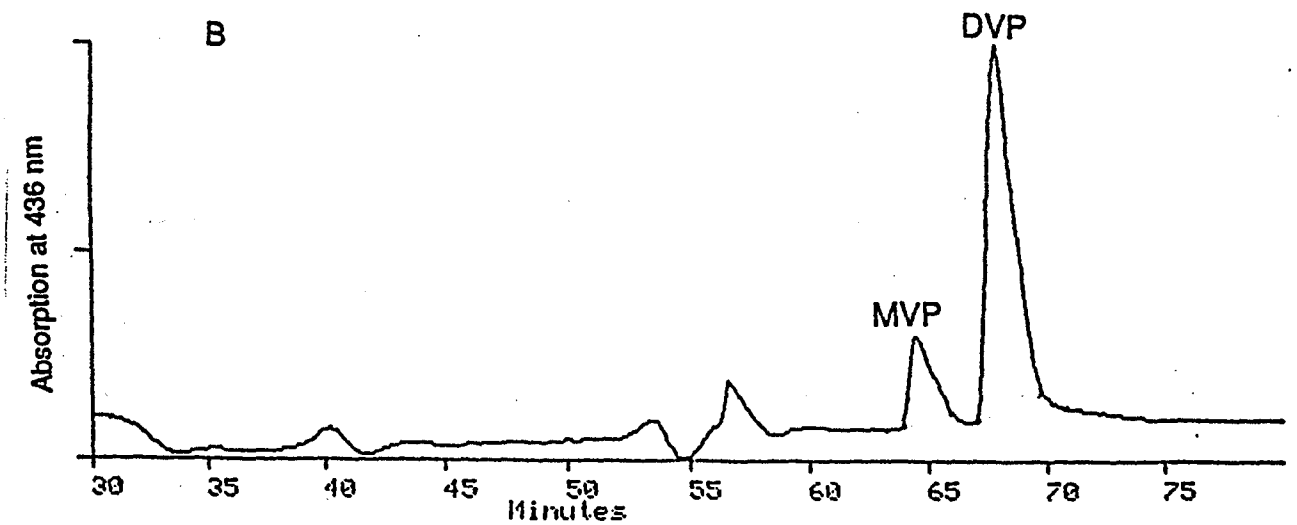
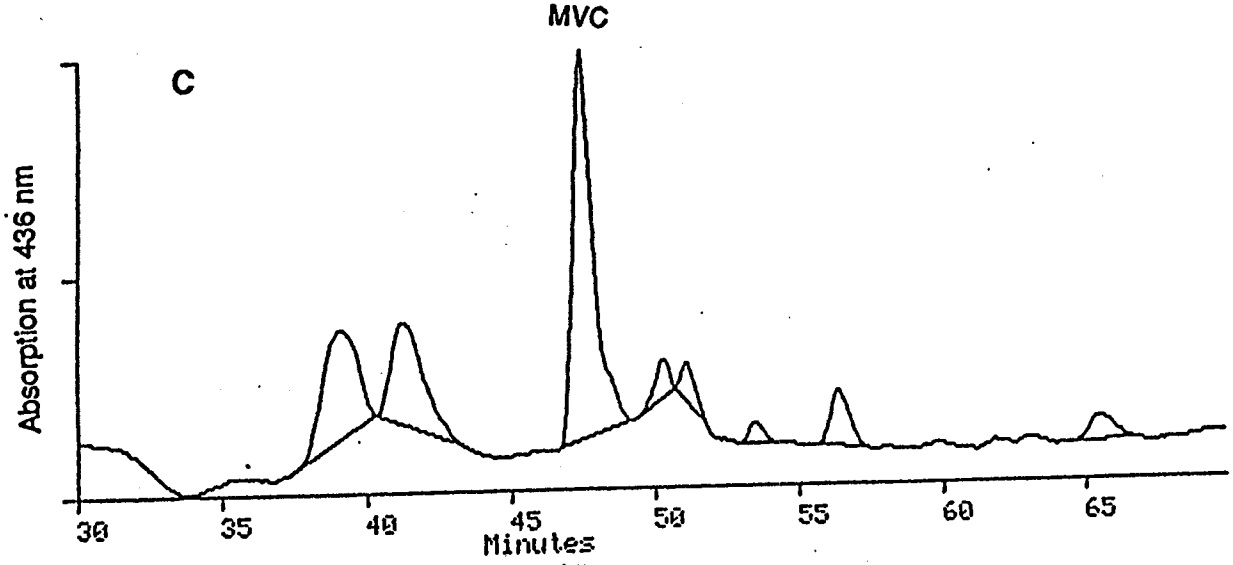

B
DVP
MVP
Absorption at 436 nm
30
35
40
45
50
55
60
65
70
75
Minutes

Fig. 3.3.2.

HPLC elution profile of MVC prepared from and oat (C) and spinach (D). Samples were analysed by the new HPLC system described in 2.3.2. and monitored at 436 nm.


MVC
C
Absorption at 436 nm
30
35
40
45
50
55
60
65
Minutes

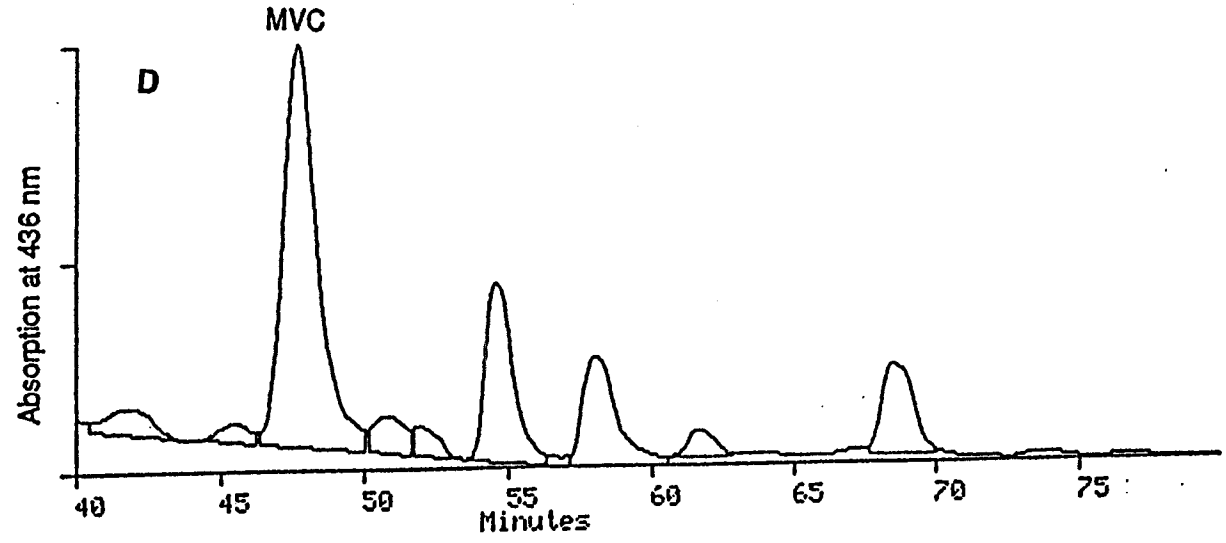
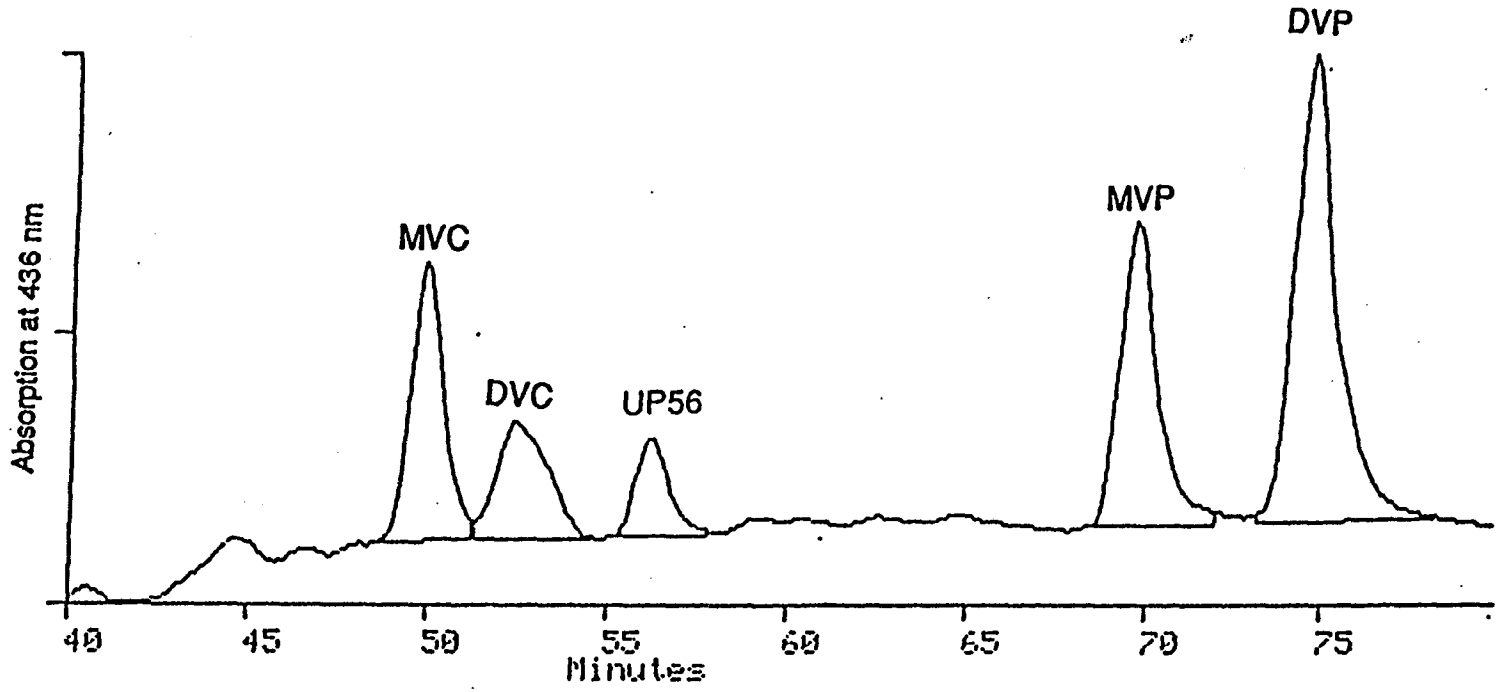

MVC
D
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes

Fig. 3.3.3.

HPLC elution profile of MVP, DVP, MVC and DVC. Two samples taken from Table 3.5.1. (**g** and **m**) were mixed together and analyzed by the new HPLC system described in 2.3.2 and monitored at 436 nm.


DVP
MVP
MVC
DVC
UP56
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes

3.4 WHEAT ENZYME ASSAYS.

Table 3.4.1 summarizes the results of the activity of either 4VR or PCR in wheat etioplasts and lysed wheat etioplast membranes using the exogenous pigment MVP:DVP (1:4) (Chapter 2.4). Our results indicated that exogenous DVP was not a substrate for the 4VR. On the basis of these results, therefore, it was apparent that we needed to generate DVC (another 4VR substrate) *in situ* by the light-requiring enzyme PCR. However, since wheat is a monovinyl plant, it accumulated predominantly MVP in the dark. Furthemore, our results suggested that only endogenous MVP was taken as a PCR substrate, while the exogenous pigments were not very likely accepted by the enzyme. Therefore, we were unable to generate the possible 4VR substrate DVC, from exogenous DVP by light activation of PCR. Hence, in the following experiments, we decided to only use the endogenous pigment which could be generated *in situ* by using etioplasts of the divinyl plant, cucumber.

TABLE 3.4.1 Relative proportion of the pigment intermediates calculated from HPLC peak areas, after different light treatments using wheat enzymes.

WHEAT SAMPLE	LIGHT TREATMENT*	INCUBATION TIME [min.]	MVC [%]	DVC [%]	MVP [%]	DVP [%]
[A] ETIOPLASTS + NADPH		at 25°C				
a	D	0 [substrate]	/	/	15	85
a_1	D	30 [only in buffer]	/	/	15	85
b	5L	0	82	18	40	60
c	5L	15	88	12	31	69
d	5L	30	100	0	36	64
[B] LYSED ETIOPLASTS + NADPH		at 25°C				
e	D	0 [only in buffer]	/	/	22	78
f	D	1	/	/	62	38
g	D	5	/	/	62	38
h	D	15	/	/	65	35
i	D	60	/	/	62	38
[C] LYSED ETIOPLASTS + NADPH		at 4°C				
j	D	0 [substrate]	/	/	20	80
k	D	0 [substrate + inactivated etioplast]	/	/	32	68
l	D	0.5	/	/	24	76
m	D	30	/	/	24	76
[D] LYSED ETIOPLASTS		at 25°C				
n	D	0 [substrate]	/	/	20	80
o	22L	0	/	/	48	52
p	22L	20	/	/	54	45
[E] LYSED ETIOPLASTS + NADPH		at 25°C				
q	D	10	41	59	30	70
s	16L+3	10	57	43	28	72

Samples were kept in the dark (D) or were exposed to X light flashes (XL) followed by dark periods of either 20s (A) or 10s (D&E); in sample E three fast light flashes immediately preceeded incubation. The standard deviation for calculation of the relative proportion of the protochlorophyllide pool is ± 1%.

3.5. OBSERVATION OF PCR- AND 4VR- ENZYME ACTIVITIES IN WHOLE ETIOPLASTS AND LYSED ETIOPLAST MEMBRANES OF CUCUMBER.

Table 3.5.1 summarizes the results of the 4VR- and PCR-enzyme activitlies in whole and lysed cucumber etioplasts described in Chapters 2.5.1 and 2.5.2, respectively. In both cases the etiolated cucumber cotyledons were given 3 light/dark treatments after harvesting in order to maximize DVP accumulation and minimize MVP contamination in experiments A - C. Isolated whole cucumber etioplasts were subsequently exposed to different brief light treatments, in order to generate DVC from DVP by the light - requiring enzyme, PCR, as a possible substrate for the 4VR.

The absorption spectrum shown in Fig 3.5.1 shows the generation of the chlorophyllide pool at 663 nm immediately after the etioplasts had been given 1light flash in experiment (A) and 3 light flashes in experiment (B). As can be seen, the peak at 625 nm (which represents the protochlorophyllide pool) was further reduced after 3 light flashes had been given to the cucumber etioplasts (B). The results of the HPLC assay showed that approximately 50 % of the pigments of the sample consisted of the protochlorophyllide pool, however.

The fluorescence spectrum, first reported by Duggan & Rebeiz (8) and obtained during our repetition of their experiment (A), showed a significant accumulation of DVC (E458,F675) by exposure to only 1 light flash and its subsequent conversion to MVC (E447,F675) (Fig 3.5.2). On the other hand, only an insignificant accumulation of DVC and its subsequent conversion to MVC was indicated by the HPLC assay shown in Fig 3.5.3.

Alternatively, when 3 light flashes were given to whole cucumber etioplasts in experiment B, the fluorescence assay indicated no significant accumulation of DVC . Therefore, it was not possible to observe its conversion to MVC by this

TABLE 3.5.1 Relative proportion of the pigment intermediates calculated from HPLC peak areas, using cucumber enzymes.

Cucumber sample	Light treatment	Incubation time [min]	MVP [%]	DVP [%]	MVC [%]	DVC [%]	P* [%]	C* [%]
[A] etioplasts								
a	D**	30	24	76	/	/	100	/
b	1L***	0	23	77	75	25	75	25
c	1L	30	30	70	86	14	70	30
[B] etioplasts								
d	D	30	31	69	0	0	87	13
e	3L****	0	40	60	32	68	50	50
f	3L	1	35	65	64	36	51	49
g	3L	3	40	60	49	51	46	54
h	3L	30	41	59	80	20	50	50
[C] lysed etioplasts + NADPH & NADH								
i	D	0	24	76	0	0	100	/
j	D	30	32	68	0	100	90	10
k	3L	0	29	71	38	62	70	30
l	3L	1	30	70	34	66	67	33
m	3L	3	30	70	77	23	68	32
n	3L	30	30	70	?	?	?	?

* The amount of the protochlorophyllide pool relative to the chlorophyllide pool, was calculated by using the equation:

$$P[\%] = \frac{A_p / 289}{A_p / 289 + A_c / 131} \times 100$$

where A_p and A_c are the HPLC areas of the protochlorophyllide and chlorophyllide pools and 289 mM^{-1} cm^{-1} and 131 mM^{-1} cm^{-1} are extinction coefficients of the protochlorophyllide and chlorophyllide pools, respectively.

** D = samples were kept in the dark.

***1L = samples were exposed to 1 light flash before dark incubation

**** 3L = samples were exposed to 3 flashes of light (with 20 s of dark between the first 2 light pulses) and incubated in the dark immediatly after the 3rd light pulse.

The standard deviation for calculation of the relative proportion of the protochlorophyllide and chlorophyllide pools is ±1%.

method (Fig 3.5.4). On the other hand, the HPLC assay shown in Fig 3.5.5 revealed a significant accumulation of DVC and its subsequent conversion to MVC. This figure clearly demonstrated reduction of 48 % of the 4- vinyl groups during the 30 min dark incubation period, using endogenous coezymes in whole cucumber etioplasts.

Fig 3.5.6 shows the fluorescence emission spectra of etioplast samples employing the excitation wavelength of DVC (458 nm). This assay shows the accumulation of DVC in experiments using whole etioplasts (A,B) and lysed etioplast membranes (C) immediately after the light treatments by the increase in an emission peak at 677-680 nm. The fluorescence maximum of this peak should be due mainly to DVC, and was seen to be greatly reduced in experiments with whole etioplasts (A, B) during a subsequent dark incubation. In the experiment with lysed etioplast membranes (C), the amount of DVC decreased much less relative to the emission peak at 630 nm (characterizing the protochlorophyllide pool). The emission maximum of MVC (E447,F675) did not significantly change relative to the peak at 629 nm during the dark incubation in all cases A - C. (Fig 3.5.7). Because one would expect decreasing levels of DVC to increase the relative amount of MVC, we were unable to explain this result.

Experiment B (where whole etioplasts were exposed to 3 light flashes) clearly demonstrated the reduction of the 4-vinyl group using endogenous coenzymes for reduction . It was not clear at this time whether the reductase enzyme required NADH or NADPH.

Therefore, in experiment C, whole cucumber etioplasts were lysed in order to eliminate the endogenous coenzymes, and exogenous coenzymes (NADPH and NADH) were added to the lysed etioplasts.

The fluorescence assay shown in Fig 3.5.8 reveals a significant accumulation of DVC (E458, F675) after 3 light flashes. But 30 min of dark incubation

produced a compound with a fluorescence excitation maximum of 451 nm. This is a 4 nm higher wavelength than expected for MVC (E447, F675).

However, the HPLC assay (Fig 3.5.9) showed that the generation of DVC by light in lysed etioplasts (C) was not as significant in comparison to whole etioplasts (B). Furthemore, in the lysed etioplasts (C), the ratio of accumulated MVC to DVC stayed the same after 1 min of dark incubation. However, after 3 min of dark incubation, 4VR activity increased the relative amount of MVC by 41%. This result demonstrated that the 4VR employed DVC and either NADPH or NADH during the first 3 min of dark incubation. After 30 min of dark incubation, a large multi - peak complex accumulated in the region of the chlorophyllide pool with a retention time maximum at 48 min, and a shoulder at a retention time of 51min. These two retention times correspond to MVC and DVC, respectively. However, for unknown reasons, the MVC and DVC did not separate clearly. This could have been due to interference by the accumulation of an unknown intermediate. (Therefore, this multi-peak complex was isolated and it exhibited a fluorescence maximum of MVC (E447,F675) in ether at 77^{o}K.)

Fig 3.5.1.

The absorption spectra in ether of pigments isolated from cucumber etioplasts showing the generation of the chlorophyllide pool (at 663 nm) by either 1 light flash (1L) (sample b) or 3 light flashes (3L) (sample e) from the protochlorophyllide pool (at 625 nm) generated in the dark (D) (sample a). Samples a, b , e are taken from Table 3.5.1.

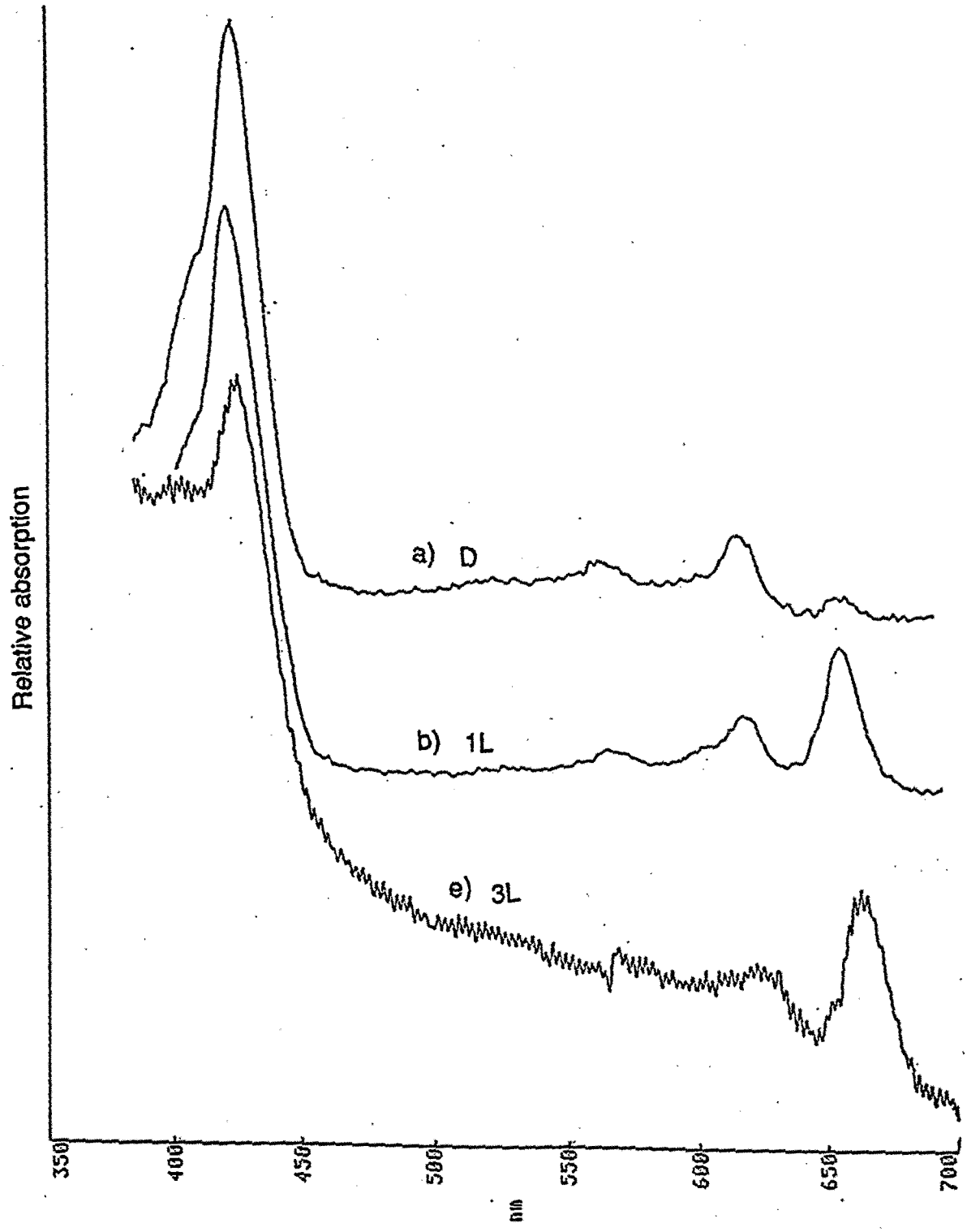

Relative absorption
a) D
b) 1L
e) 3L
350
400
450
500
550
600
650
700
nm

Fig 3.5.2.

Fluorescence excitation spectra of pigments isolated from whole cucumber etioplasts exposed to 1 light flash.

The fluorescence excitation spectra in ether at 77^{o}K of the emission of the chlorophyllide pool from experiment (A). The DVP was converted into DVC by 1 light flash (b) and after 30 min of dark incubation resulting in the conversion of DVC (E458,F675) to MVC (E447,F675) (c).

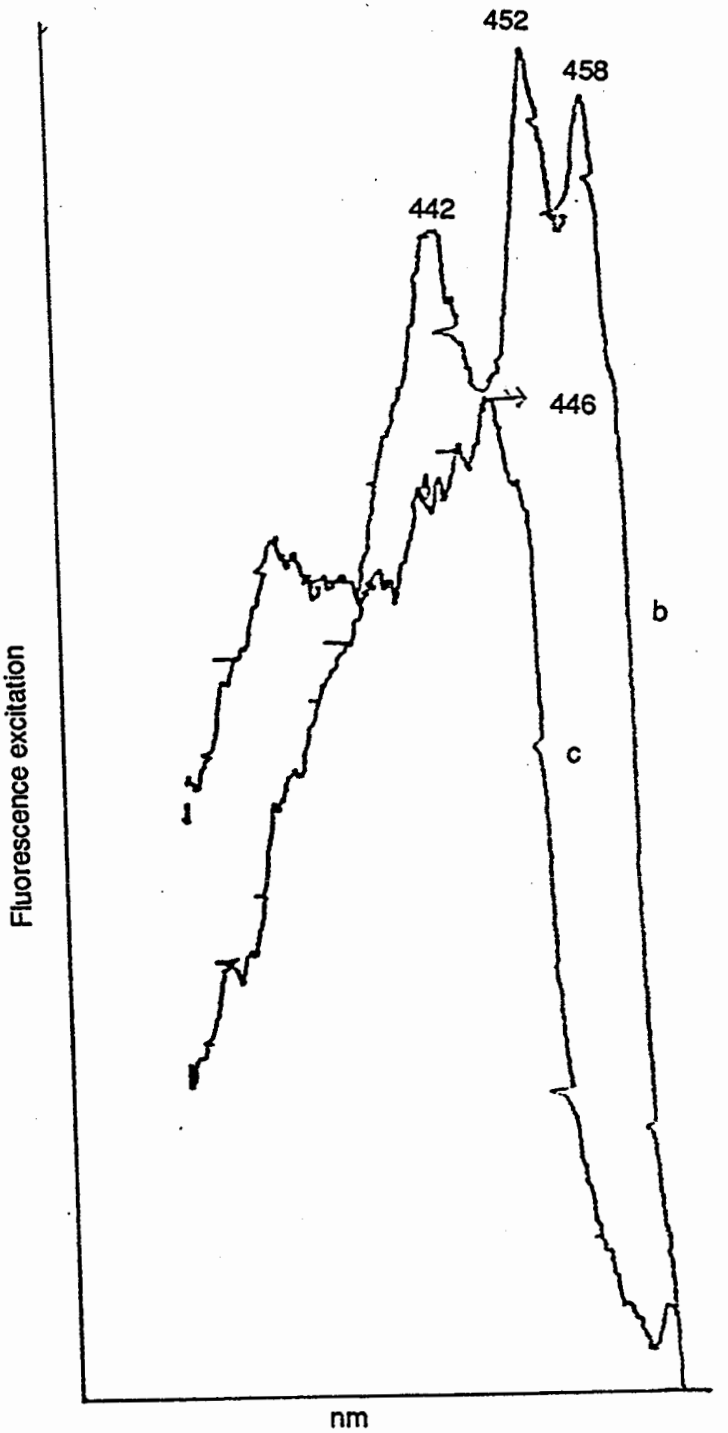
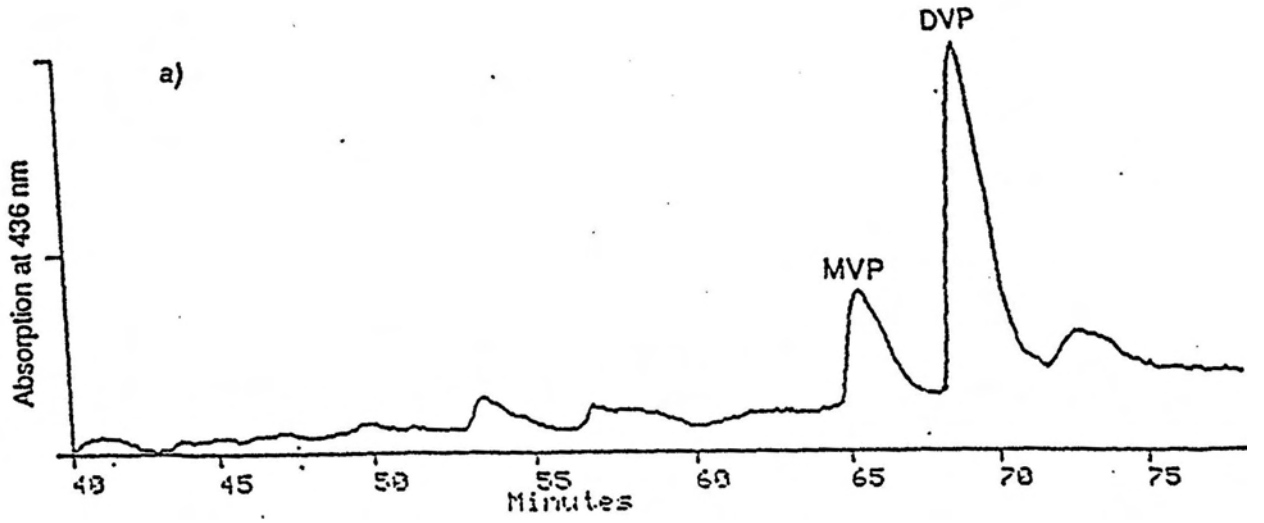
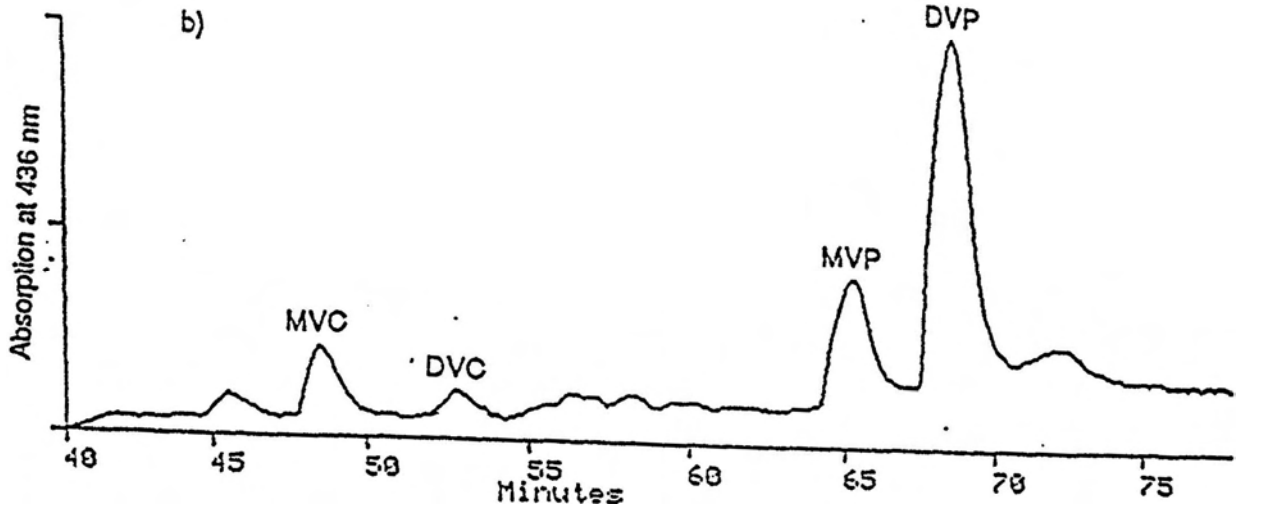

452
458
442
446
b
c
Fluorescence excitation
nm

Fig 3.5.3

HPLC elution profile of pigments isolated from whole cucumber etioplasts exposed to 1 light flash.

The HPLC elution profile of protochlorophyllide and chlorophyllide pools from experiment A monitored at 436 nm: (a) the dark control; (b) immediately after the flash; and (c) after 30 min of dark incubation. The corresponding peaks are labeled.


a)
DVP
MVP
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes


b)
DVP
MVP
MVC
DVC
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes

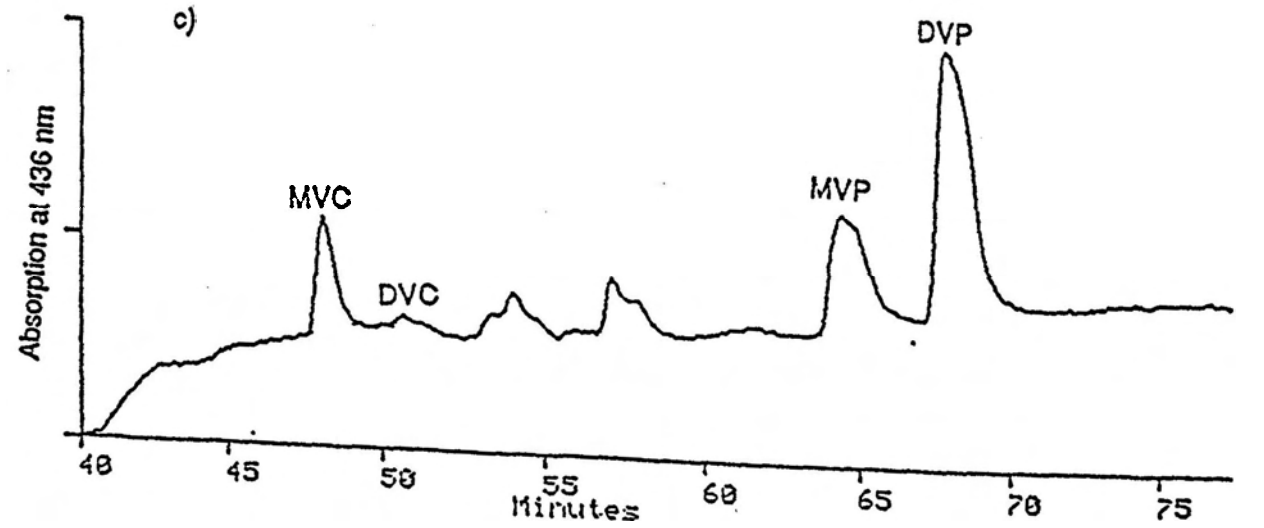

c)
DVP
MVP
MVC
DVC
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes

Fig 3.5.4

Fluorescence excitation spectra of pigments isolated from whole cucumber etioplasts exposed to 3 light flashes.

The fluorescence excitation spectra in ether at 77°K of the emission of the chlorophyllide pool at 675 nm from experiment B. The DVP was converted into DVC (E458,F675) by 3 light flashes: (e) immediately after the flashes; and after the incubation in the dark for (f) 1min, (g) 3 min, and (h) 30 min

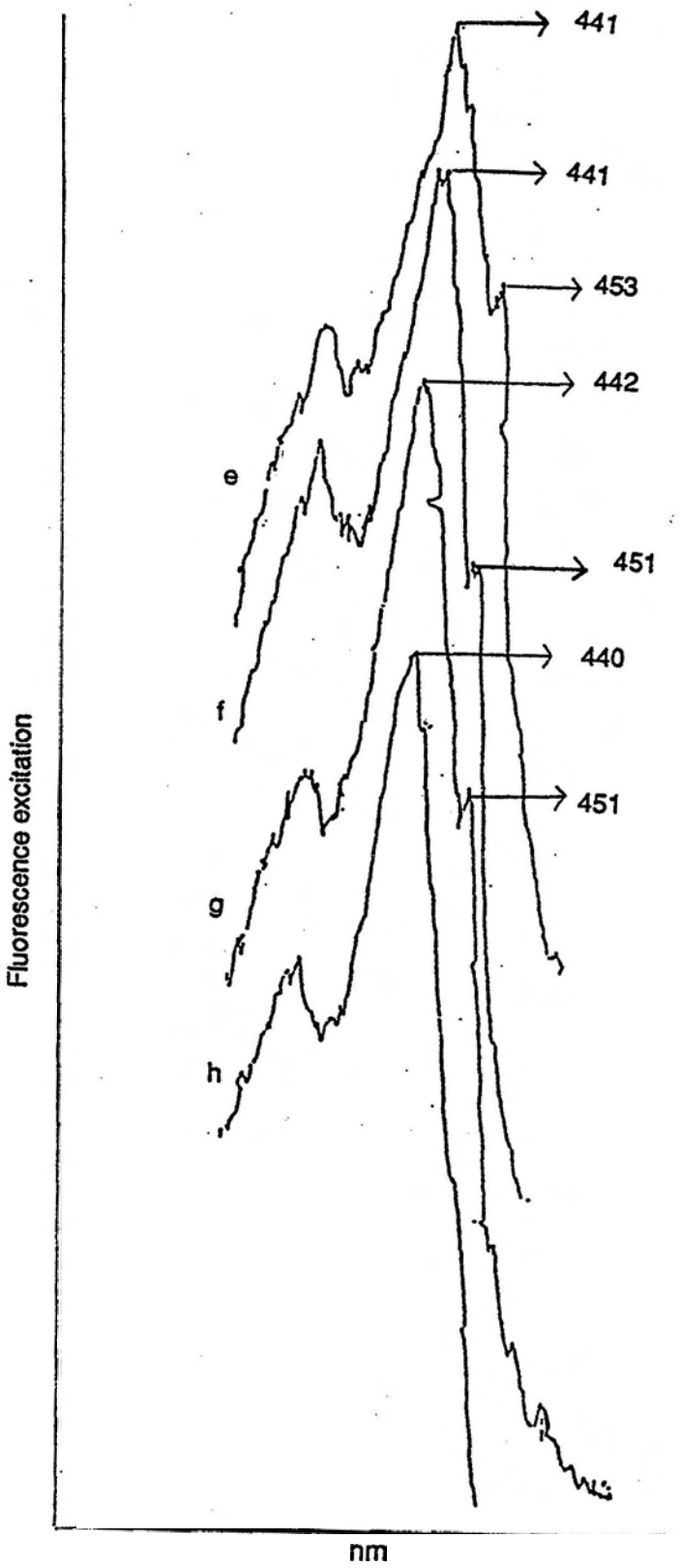

441
441
453
442
e
451
440
f
451
g
h
Fluorescence excitation
nm

Fig 3.5.5

HPLC elution profile of pigments isolated from whole cucumber etioplasts exposed to 3 light flashes.

The HPLC elution profile of protochlorophyllide and chlorophyllide pools from experiment B monitored at 436 nm: (d) the dark control; (e) immediately after 3 light flashes; and after (f) 1 min, (g) 3 min, and (h) 30 min of dark incubation. The corresponding peaks are labeled.

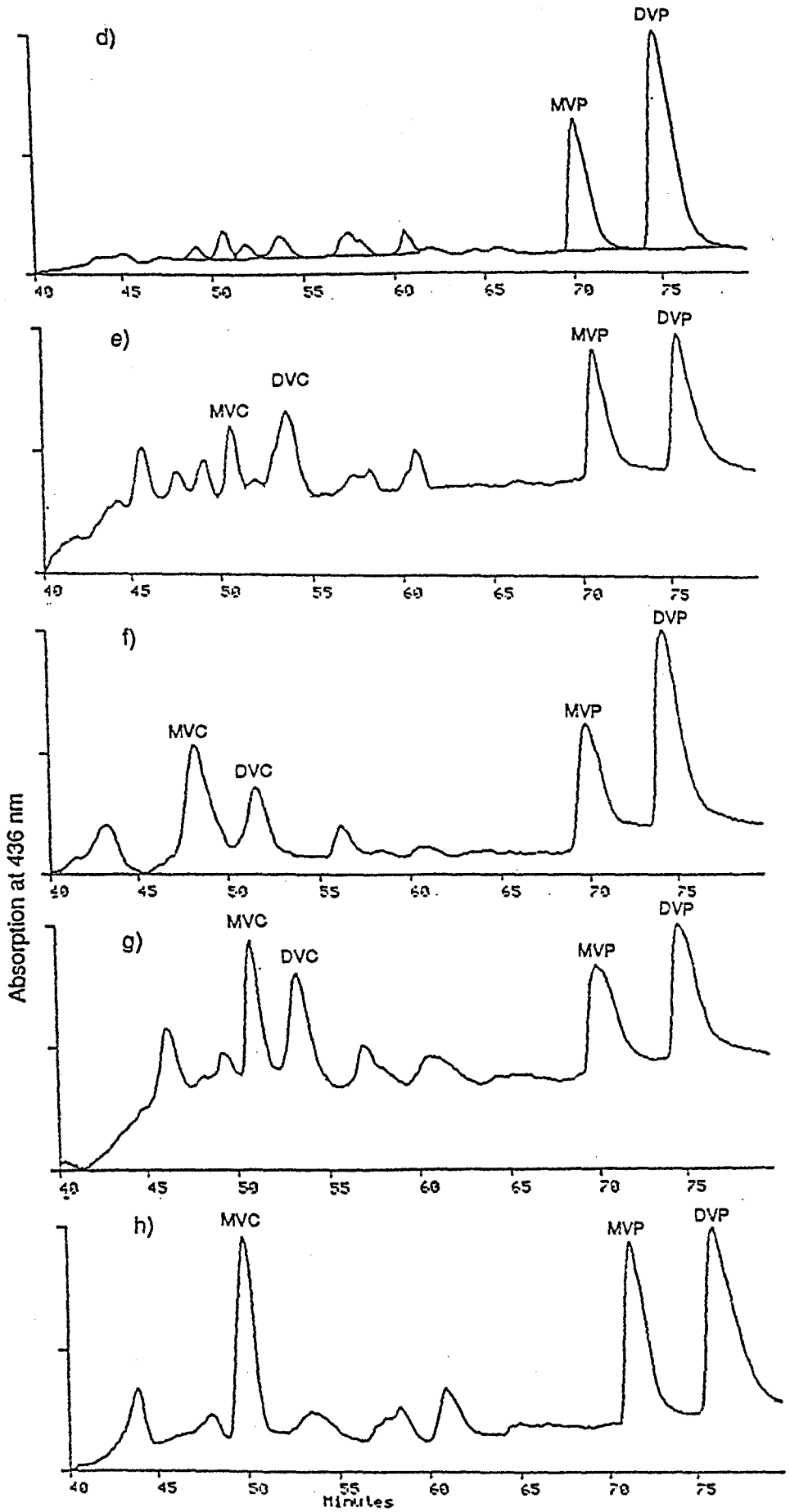
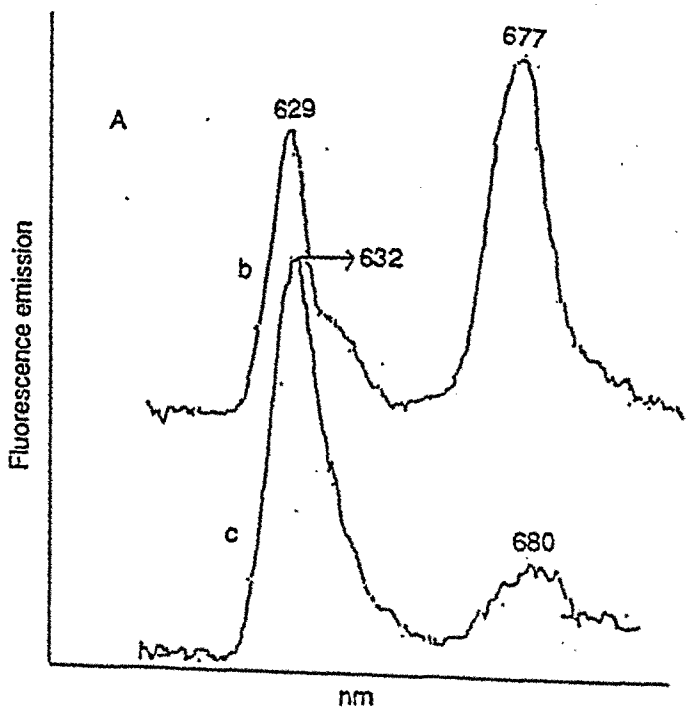
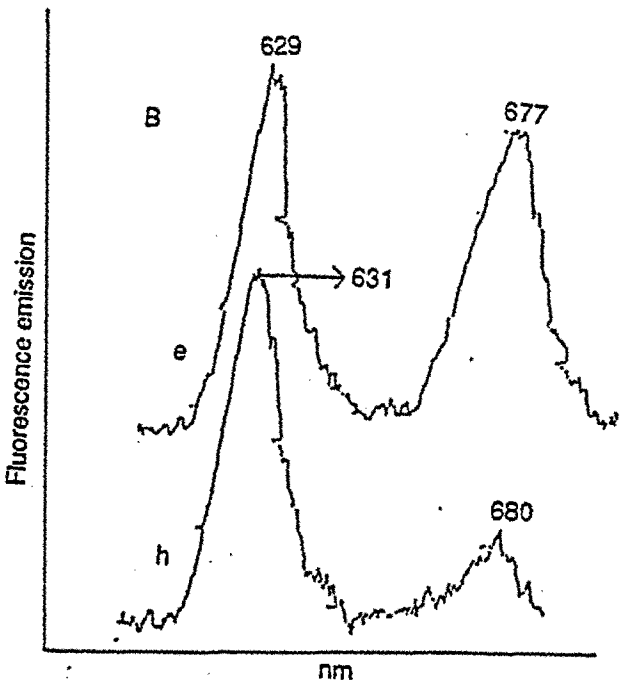

d)
DVP
MVP
40
45
50
55
60
65
70
75
e)
MVP
DVP
DVC
MVC
f)
DVP
MVP
MVC
DVC
g)
MVC
DVC
MVP
DVP
h)
MVC
MVP
DVP
Absorption at 436 nm
Minutes

Fig.3.5.6.

Fluorescence emission spectra of DVC.

The fluorescence emission spectra of DVC (E458,F675) in ether at 77°K generated by excitation monitored at 458 nm. DVC (E458,F675) was generated from DVP in either whole etioplast (A, B) or lysed etioplast membranes (C) by either 1 light flash (A) or 3 light flashes (B, C): (b,e,k) immediately after the light flash (s); and (c,h,n,) after 30 min of dark incubation.


A
629
677
b
632
c
680
Fluorescence emission
nm


B
629
677
631
e
h
680
Fluorescence emission
nm

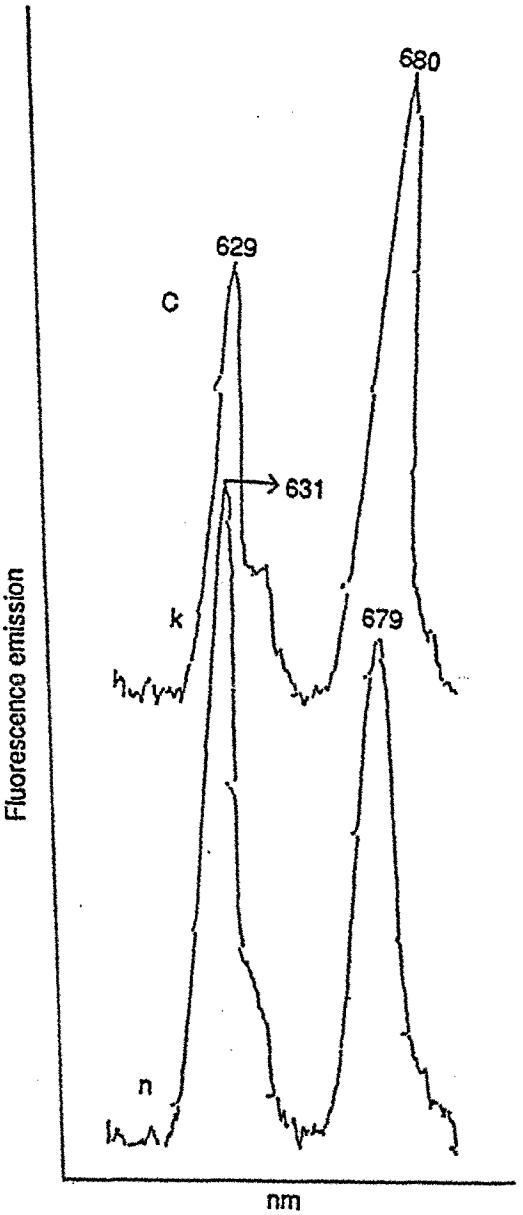
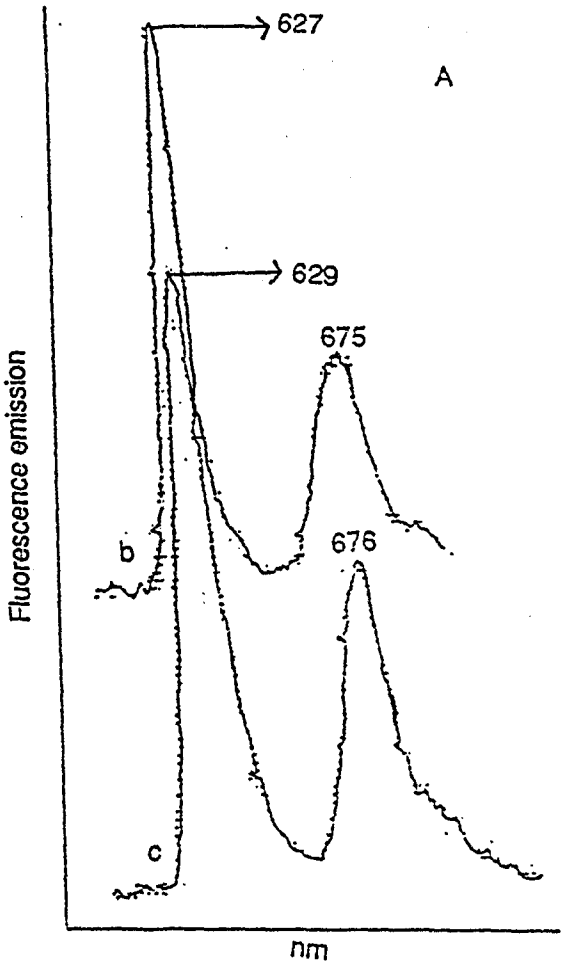
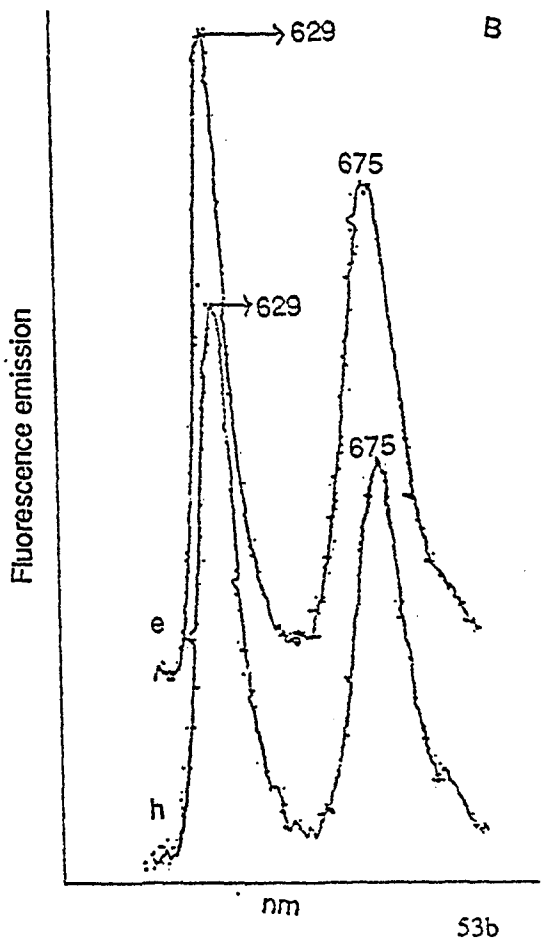

C
629
680
631
k
679
n
Fluorescence emission
nm

Fig 3.5.7

Fluorescence emission spectra of MVC.

The fluorescence emission spectra of MVC in ether at 77^{o}K generated by excitation monitored at 447 nm. The fluorescence emission of MVC (E447,F675) was generated in either whole etioplasts (A, B) or lysed etioplast membranes (C) by either 1 light flash (A) or 3 light flashes (B, C): (b,e,k) immediately after the light flashes; and (c,h,n,) after 30 min of dark incubation.


627
A
629
675
b
676
c
Fluorescence emission
nm


629
B
675
629
675
e
h
Fluorescence emission
nm

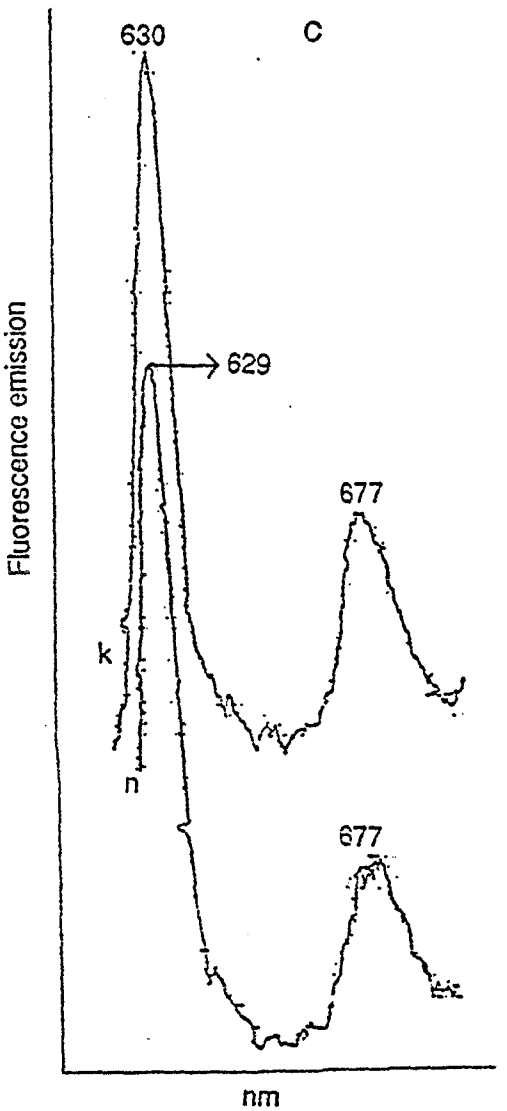

630
C
629
677
k
n
677
Fluorescence emission
nm

Fig 3.5.8

Fluorescence excitation spectra of pigments isolated from lysed cucumber etioplast membranes exposed to 3 light flashes.

The fluorescence excitation spectra in ether at 77°K of the emission of the chlorophyllide pool monitored at 675 nm from experiment C. The DVP was converted into DVC (E458,F675) by 3 light flashes: (k) immediately after the light flashes; and incubated in the dark for (l) 1 min, (m) 3 min, and (n) 30 min. The incubation resulted in the accumulation of a compound giving a fluorescence maximum at 451 nm.

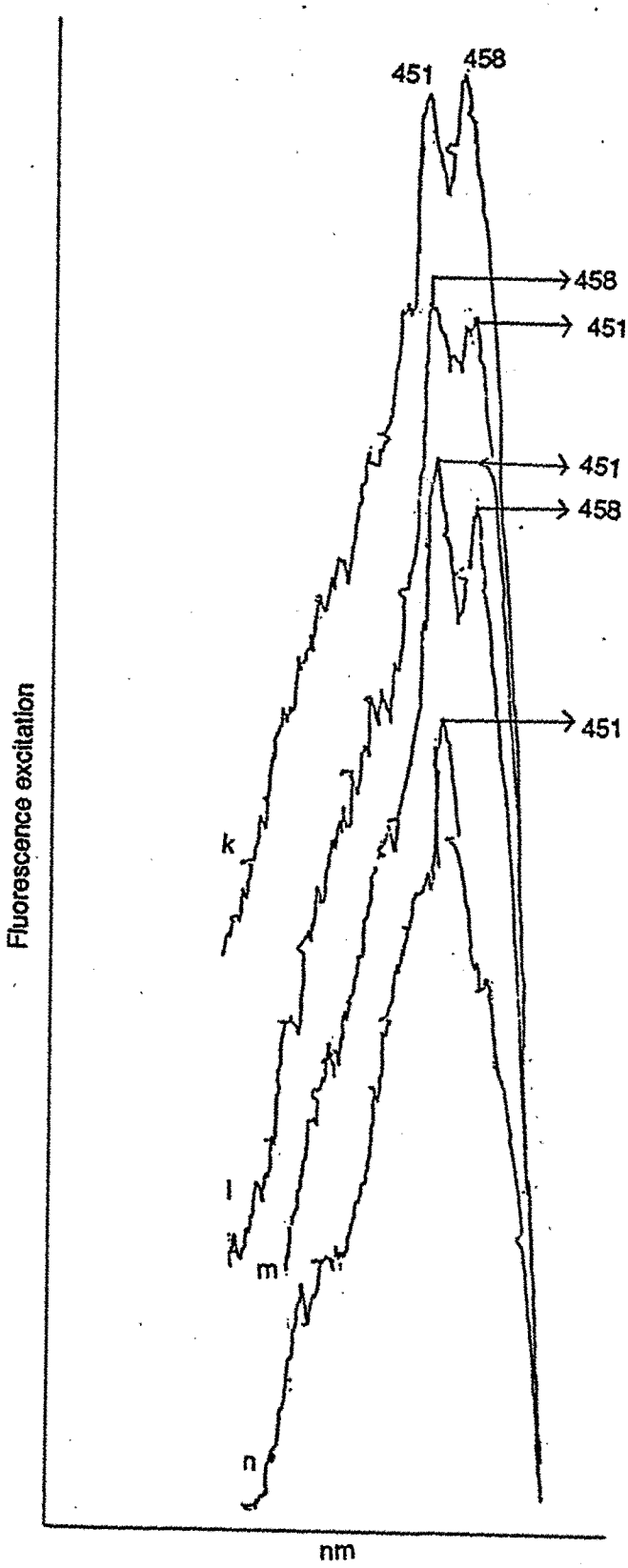
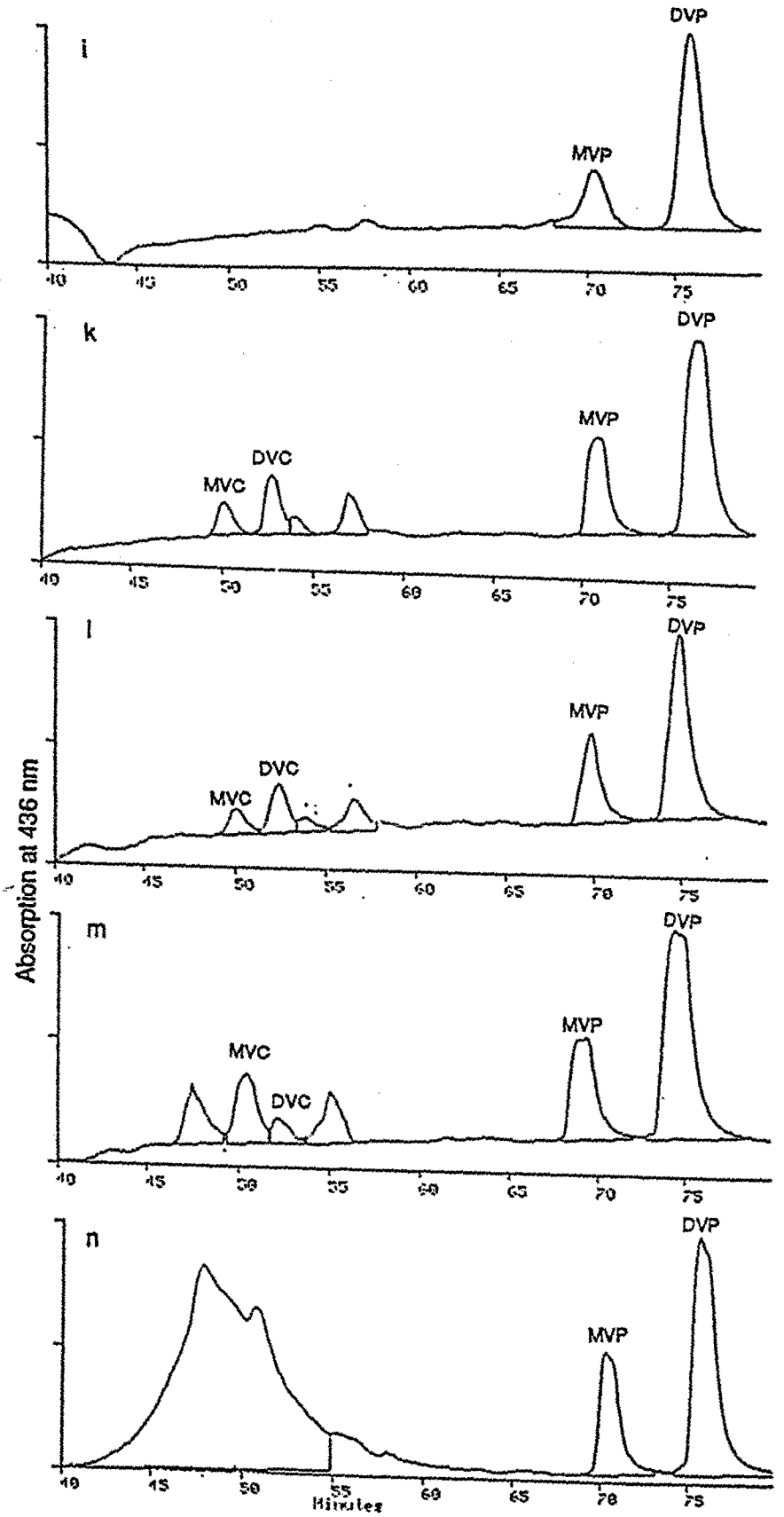

451
458
458
451
451
458
451
k
l
m
n
Fluorescence excitation
nm

Fig 3.5.9

HPLC elution profile of pigments isolated from lysed etioplast membranes exposed to 3 light flashes.

The HPLC elution profile of protochlorophyllide and chlorophyllide pools from experiment C monitored at 436 nm; (i) the dark control; (k) immediately after the flashes; and after incubation in the dark for (l) 1min, (m) 3 min, and (n) 30 min. The corresponding peaks of interest are labeled.


i
DVP
MVP
40
45
50
55
60
65
70
75
k
DVP
MVP
DVC
MVC
l
DVP
MVP
DVC
MVC
Absorption at 436 nm
m
DVP
MVP
MVC
DVC
n
DVP
MVP
Minutes

3.6. THE ISOLATION AND VERIFICATION OF THE HPLC PEAKS CORRESPONDING TO PIGMENTS IN THE CHLOROPHYLLIDE AND PROTOCHLOROPHYLLIDE POOLS.

Table 3. 6. 1 summarizes the fluorescence excitation and emission maxima of the pigments purified by HPLC on powdered polyethylene column (compared with the published values). Our purified MVC and DVC (Fig 3.6.2 and Fig 3.6.4) exhibited slightly different excitation maxima when compared to published values. The latter were recorded in a mixture of several tetrapyrroles. It seems that the presence of the other tetrapyrroles in the mixture shifts the wavelength either higher or lower, depending on the relative proportion of both tetrapyrroles.

For example, the fluorescence excitation wavelength of DVC (Fig 3.6.4) is 3 nm higher compared to published values, due probably to the absence (or presence of only very small amounts) of MVC in the sample. On the other hand, Fig 3.6.2 shows a 1 nm lower excitation wavelength compared to the published value for DVC due to the presence of some MVC.

The fluorescence excitation wavelength of our purified MVC sample was either 4 nm (Fig 3.6.4) or 2 nm higher (Fig 3.6.2) than the published value. This may be explained by the presence of an unknown compound with an excitation maximum at a higher wavelength (E469,F675). This is seen clearly in Fig 3.6.4.

Both the HPLC - purified MVP [E439,F627] and DVP [E443, 452,F627] exhibited similar excitation wavelength maxima to published data when monitored by emission at 625 nm (Fig 3.6.1). A surprising result relating to the purified MVP and DVP is that they also exhibited emission at either 670 nm or at 673 nm when excited at 437 nm or 443 nm respectively. This emission has previously been observed only by chlorins (dihydroporphyrins). Therefore, it is possible that the fractions contain unknown compounds (chlorins) with similar

HPLC properties which may also participate in the biosynthetic pathway of chlorophyll *a*. Further study is required to determine any possible functions for these compounds.

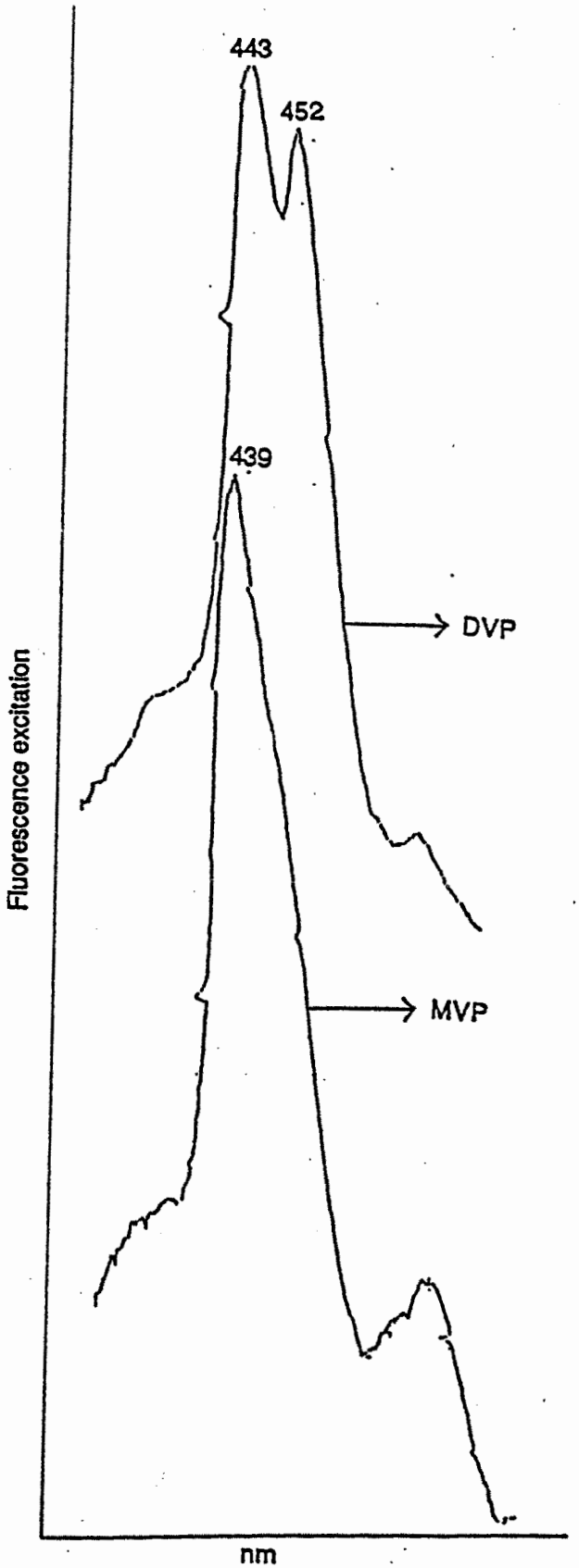
The unknown peak labeled UP56 with a retention time of 56 min is present in all samples containing protochlorophyllide and chlorophyllide pools (Fig 3.3.3 and Fig 3.6.3).The other unknown peak (UP1) with a retention time of 5-12 min is contained mainly in the pigments isolated after light treatment of whole or lysed etioplasts. Both also exhibit fluorescence maxima which are included in **Table 3.6.1**.

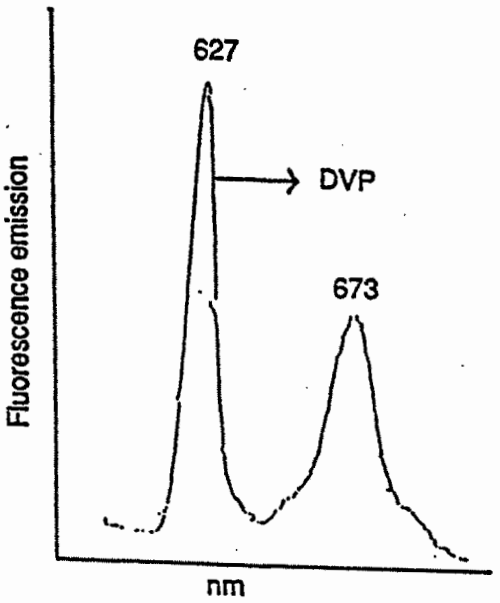
Table 3.6.1 The fluorescence excitation (E) and emission (F) maxima of HPLC purified tetrapyrroles at 77°K in ether.

Tetrapyrroles	published fluorescence maxima	fluorescence maxima of purified pigments from Fig 3.3.3	HPLC retention time of the pigments from Fig 3.3.3 [min].	fluorescence maxima of purified pigments from Fig 3.6.3	HPLC retention time of the pigments from Fig 3.6.3 [min].
MVC	(E447,F675) (22)	(E449,F675)	49.9	(E451,469,F673)	49.6
DVC	(E458,F675) (30)	(E449,457,F675)	52.5	(E461,F677)	51.9
MVP	(E437,F625) (19)	(E439,F627)	69.7	/	69.2
DVP	(E443,451,F625) (22)	(E443,452,F627)	74.7	/	74
UP1	unknown	(E440,F680)	5-12	/	5-12
UP56	unknown	(E440,F625,670)	56	(E440,F625,670)	56

Fig 3.6.1

Fluorescence spectra in ether at 77^{o}K of MVP and DVP purified on HPLC shown in Fig 3.3.3. Fluorescence excitation maxima were monitored at 625 nm for both MVP and DVP , while fluorescence emission maxima were excited at 443 nm for DVP and at 437 nm for MVP.


443
452
439
DVP
MVP
Fluorescence excitation
nm


627
DVP
673
Fluorescence emission
nm

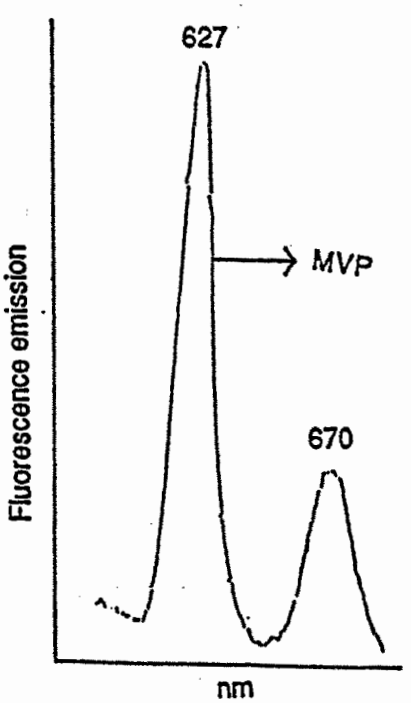
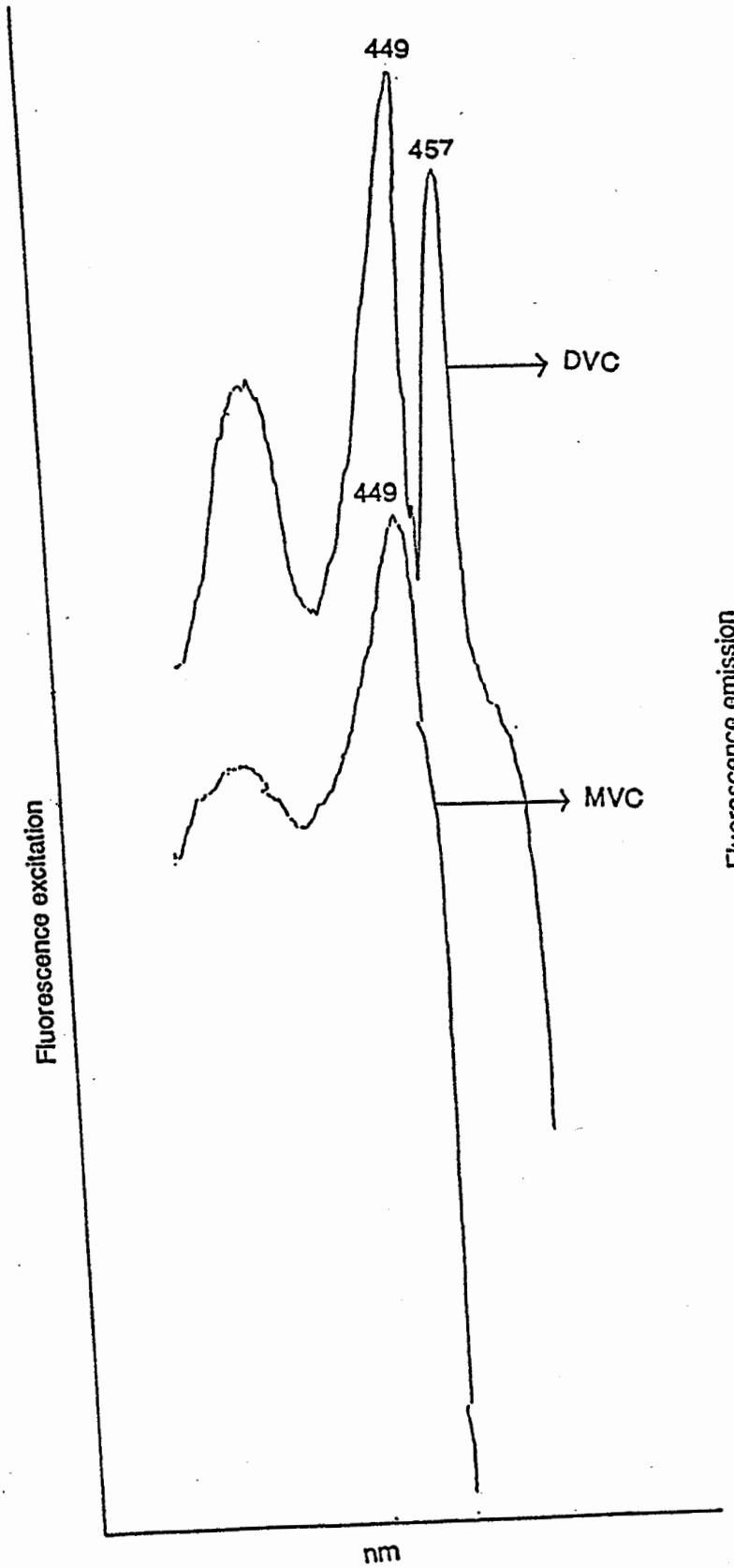

627
MVP
670
Fluorescence emission
nm

Fig 3.6.2

Flurescence spectra in ether at 77°K of MVC and DVC purified by HPLC as shown in Fig 3.3.3. Fluorescence excitation of MVC and DVC were monitored at the emission maximum of 675 nm, while fluorescence emission maxima were excited at 447 nm for MVC and at 458 nm for DVC.


449
457
DVC
449
MVC
Fluorescence excitation
nm

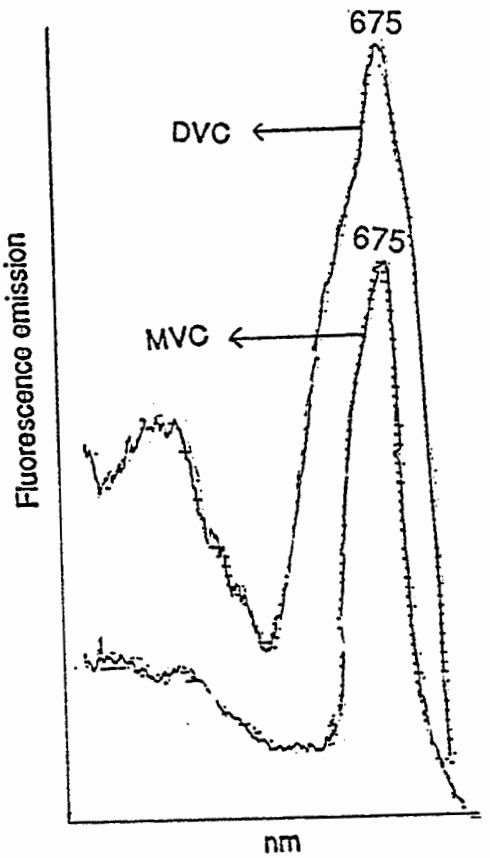

675
DVC
675
MVC
Fluorescence emission
nm

Fig 3.6.3

HPLC elution profile of MVP, DVP, MVC and DVC on a powdered polyethylene column by the gradient method described in Chapter 2.3.2. The sample was prepared by mixing samples **k** and **m** from Table 3.5.1.

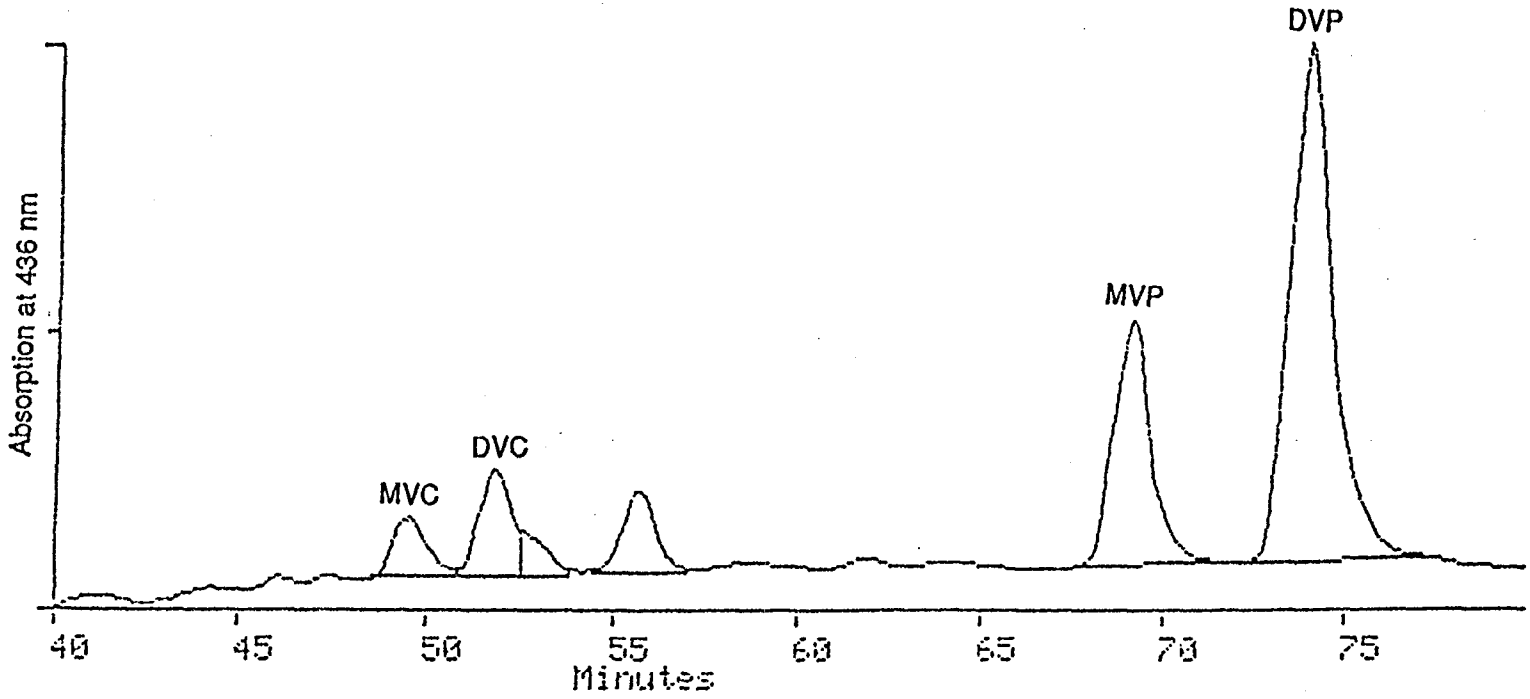
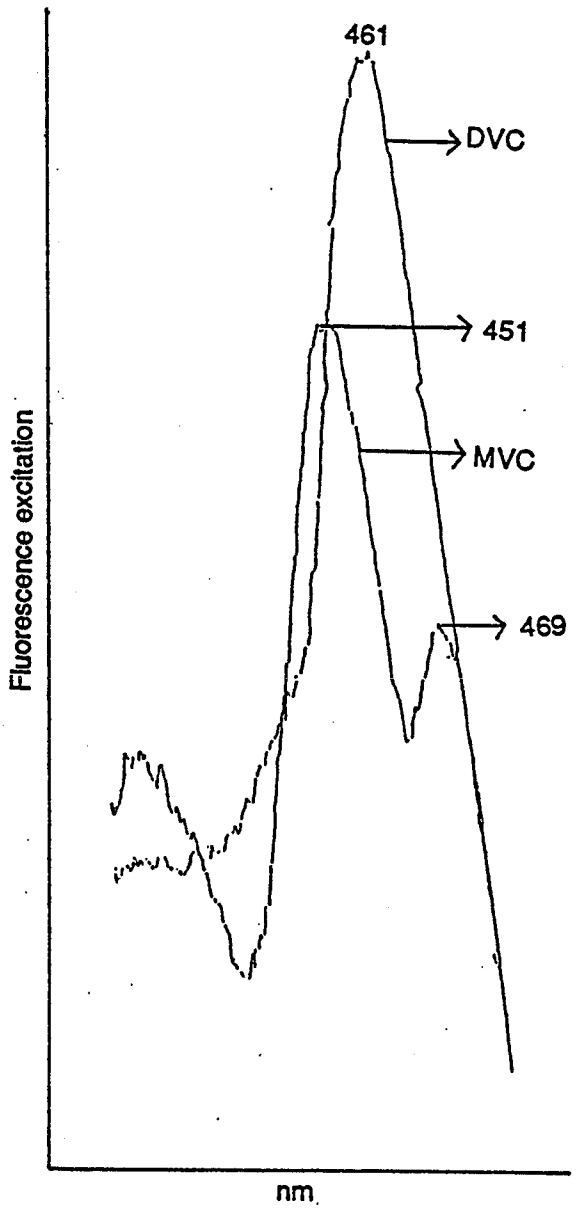
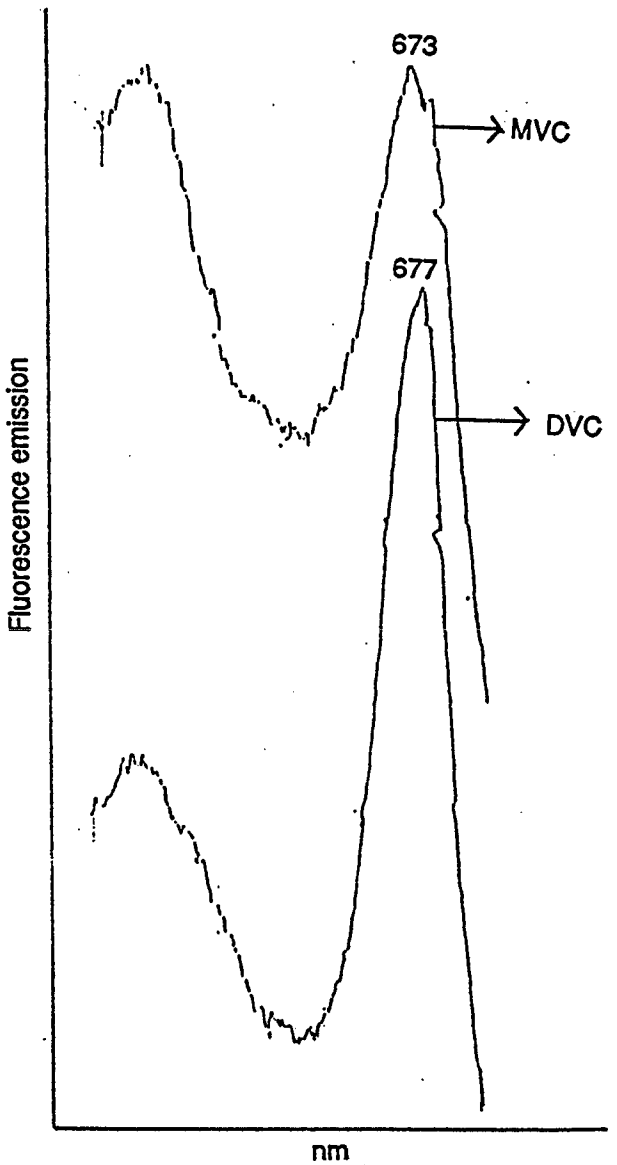

DVP
MVP
DVC
MVC
Absorption at 436 nm
40
45
50
55
60
65
70
75
Minutes

Fig 3.6.4

Fluorescence spectra in ether at 77^{o}K of MVC and DVC purified by HPLC as shown in Fig 3.6.3. Fluorescence excitation maxima of MVC and DVC were both monitored at 675 nm, while fluorescence emission maxima were excited at 458 nm for DVC and at 447 nm for MVC.


461
DVC
451
MVC
469
Fluorescence excitation
nm


673
MVC
677
DVC
Fluorescence emission
nm

3.7 THE OBSERVATION OF ACTIVITY DUE TO 4-VINYL REDUCTASE IN LYSED CUCUMBER ETIOPLAST MEMBRANES IN THE PRESENCE OF RADIOACTIVELY LABELED NICOTINAMIDE COENZYMES (THE 4R- AND 4S-ISOMERS OF [^{3}H]NADPH).

Table 3.7.1 summarizes the results of the experiment described in Chapter 2.5.4. Etioplast membranes were isolated from etiolated cucumber cotyledons and then exposed to 6 light flashes in order to generate the 4VR substrate, DVC, from DVP. Either unlabeled NADH or the 4R- or 4S-isomer of [^{3}H] NADPH was added immediately after the last light flash. The conversion of the fluorescence excitation maximum of DVC (E454,F675) to that of MVC (E449,F675) during the 10 min dark incubation following the last light flash is shown in Fig 3.7.1. This conversion can be seen most clearly after the addition of [4R-^{3}H]NADPH. The detected fluorescence excitation maximum for DVC was 4 nm lower, and that for MVC was 2 nm higher, than published values. These discrepancies have already been discussed in Chapter 3.6, and may have been due to interference by other tetrapyrroles in the isolates.

Both the fluorescence spectrum and HPLC assay (Fig. 3.7.1 and 3.7.2) indicated that the accumulation of DVC was not as extensive as in experiment C (described in Chapter 3.5) in which coenzymes were added to lysed etioplasts during the light treatment. It seems that the omission of exogenously added NADPH during the light treatment reduced the extent of the photoconversion of DVP to DVC, perhaps because NADPH had dissociated from the PCR ternary complex and the DVP was no longer photoconvertible. Also the photoconversion of the protochlorophyllide pool to the chlorophyllide pool was not the same in each sample. In sample a (the dark control), the protochlorophyllide pool was present to the extent of 89% of the pigments, and

TABLE 3.7.1 The relative amount of chloroplast pigments calculated from HPLC peak areas and their absorption and fluorescence maxima.

sample	a	b	c	d	e	f
coenzyme	none	none	none	NADH	[4S-H^3] NADPH	[4R-H^3] NADPH
Light treatment	D*	6L**	6L	6L	6L	6L
Incubation time [min]	10	0	10	10	10	10
***Ratio [%]						
P	90	81	71	69	77	56
C	10	19	29	31	23	44
Ratio [%]						
MVP	43	24	25	38	41	41
DVP	57	76	75	62	59	59
Ratio [%]						
MVC	21	62	40	21	46	64
DVC	79	38	60	79	54	36
λ [max] in ether [nm]	436.4, 625	436.5, 625	437.2, 625	437.9, 625	437.3, 625	438.9, 625
Fluorescence emission at 675 nm in ether at 77K [nm]	/	444,447, 454	446,454	447,453	449,454	445,449

*D = samples were kept in the dark
** 6L = samples were exposed to 6 fast light flashes with 20 s dark intervals betwen the first 5 light pulses and incubated in the dark immediatly after the 6^{th} light pulse.
*** The amount of the protochlorophyllide pool relative to the chlorophyllide pool was calculated using the equation:

$$P[\%] = \frac{A_p / 289}{A_p / 289 + A_c / 131} \times 100$$

where A_p and A_c are the HPLC areas of the protochlorophyllide and chlorophyllide pools, and 289 $mM^{-1}cm^{-1}$ and 131 $mM^{-1}cm^{-1}$ are extinction coefficients for the protochlorophyllide and chlorophyllide pools at 436 nm, respectively.
The standard deviation for calculation of the relative amount of the protochlorophyllide and chlorophyllide pools is $\pm$1%.

the ratio of MVP:DVP was about 2:3. In sample b, little or no photoconversion of the protochlorophyllide pool had occurred. However, the MVP to DVP ratio of the protochlorophyllide pool had changed greatly (to about 1:3), indicating that some of the MVP had preferentially disappeared (in relation to DVP), perhaps either by photoconversion or photodecomposition to the band appearing at a retention time of 75 min (UP75). Such a preferential loss of MVP (and concurrent appearence of UP75) was also observed in sample c (incubation with no added coenzymes) and, to a lesser extent, in sample d (incubation with NADH) but not when incubation was carried out with NADPH (samples e & f). Sample c, d & e all exhibited a net photoconversion of about 10-15 % of the undecomposed protochlorophyllide pool, while in sample f, the net photoconversion was about 33%. The MVC:DVC ratio in sample c was about 2:3 (ie. about the same as the MVP:DVP ratio in the dark control, indicating an approximately equal photoconversion of both components. [For some reason, this was not the case in sample d, however, and there seems to have been a preferential photoconversion of DVP with respect to MVP because the MVC:DVC ratio in this sample was about 1:4]. At the end of the 10 min incubation period, the MVP:DVP ratio in samples d, e & f remained approximately the same as that of the dark control (sample a), ie. about 2:3, indicating that there had been no reduction of the 4-vinyl group at the protochlorophyllide level. In contrast, the MVC:DVC ratio in samples e & f (containing NADPH) had increased in comparison to samples c (no coenzyme) & d (containing NADH), indicating that 4-vinyl reduction was occurring in the chlorophyllide pool, and that it required NADPH. Unfortunately, the extent of 4-vinyl reduction in the presence of [4S-^{3}H]NADPH (sample e) was far less (ca.3-6%) than that in the presence of [4R-^{3}H]NADPH (sample f) (ca. 21-24%), making a comparison of the extent of the incorporation of radioactivity extremely difficult.

Table 3.7.2 and Figs. 3.7.3 and 3.7.4 summarize the results of tritium labeling experiments in lysed cucumber etioplasts containing endogenous pigment. It can be seen that approximately 7.3 times as much activity was incorporated into the extract when the 4R-isomer (rather than 4S-isomer) of [^{3}H]NADPH was used as the source of reduction. However, when the extracts were separated by HPLC and the fractions collected and counted for the presence of radioactivity, most of the radioactivity was not found in the region of known peaks. The highest level of activity in both samples was found at a retention time of 10-12 min. This unknown pigment (termed UP1) was seen at aproximately this retention time in most extracts. The spectrum of an isolated UP1 fraction exhibited absorption maxima at 596 and 631 nm, and it is possible that it was the monovinyl derivative of protoporphyrin (or its monomethyl ester) formed by the action of the 4VR on endogenous MgPME. Neither sample contained any radioactivity significantly above backround in either the protochlorophyllide pool (either MVP or DVP) or the chlorophyllide pool (either MVC or DVC), although small peaks of radioactivity were visible at 25 and 52.5 min (the latter being the retention time of DVC) in the 4S-isomer sample. Activity in the latter peak could not be due to the action of the 4VR, and could only have appeared in this compound by the action of a "dark PCR" on DVP. [In this respect it is known that the light activated PCR is specific for the 4S-isomer of NADPH (39)]. The only peak significantly above background (in addition to the UP1 peak) was located at a retention time of 42.5 min (termed the UR band) in the 4R-isomer sample, which corresponded neither to MVC nor the unknown pigment (UP46) with absorbence at 436 nm. Whatever was contained in the UR band at 42.5 min had no absorbance at 436 nm (Fig. 3.7.6), but may have had properties similar to MVC (as UP46 would have had as well) because of such a similar retention time. Athough it can be speculated that one or both of these bands may have been due to the presence

TABLE 3.7.2 Tritium assay of cucumber etioplasts.

[A] [4R-^{3}H]-NADPH	amount [nmol]	activity [nCi]	HPLC area [%]	Incorporation to pigment [%]
injected sample	0.0553	0.64	100	/
UR	?	0.175	?	27.3
UP46	?	0.056	17.86	8.75
MVC	0.0064	0	11.54	/
DVC	0.0032	0	5.79	/
MVP	0.0113	0	20.5	/
DVP	0.0157	0	28.4	/
Others	?	0.33	?	/
[B] [4S- ^{3}H] NADPH				
injected sample	0.0483	0.088	100	/
UP46	?	0	4.33	/
MVC	0.0022	0	4.61	/
DVC	0.0025	0	5.38	/
MVP	0.0144	0	29.81	/
DVP	0.021	0	43.45	/
Others	?	0.088	?	/

* The sample injected into the HPLC was 1/10 of the total extract of the lysed etioplasts containing 69 nCi (A) or 85 nCi (B).
** A blank which contained 0.035 nmol of bleached pigment was subtracted from each fraction.
*** The amount of pigment was calculated from the percentage of the HPLC peak area by using the extinction coefficients of Table 3.7.1.

of an intermediate (or intermediates) in the reduction of DVC to MVC by the 4VR which was labeled only by the 4R-isomer (and not the 4S-isomer) of [^{3}H]NADPH (*cf.* Discussion), we have no additional information of the nature of either peak.

Fig 3.7.1

Fluorescence excitation spectra of MVC and DVC after various treatments described in Table 3.7.1. Spectra were recorded in ether at 77°K monitored at the emission maximum of 675 nm, recorded either (a) immediately, or (b,c, and f) 10 min after the photoconversion. Samples contained either (b) no coenzyme, (e) [4S-^{3}H]NADPH, or (f) [4R-^{3}H]NADPH.

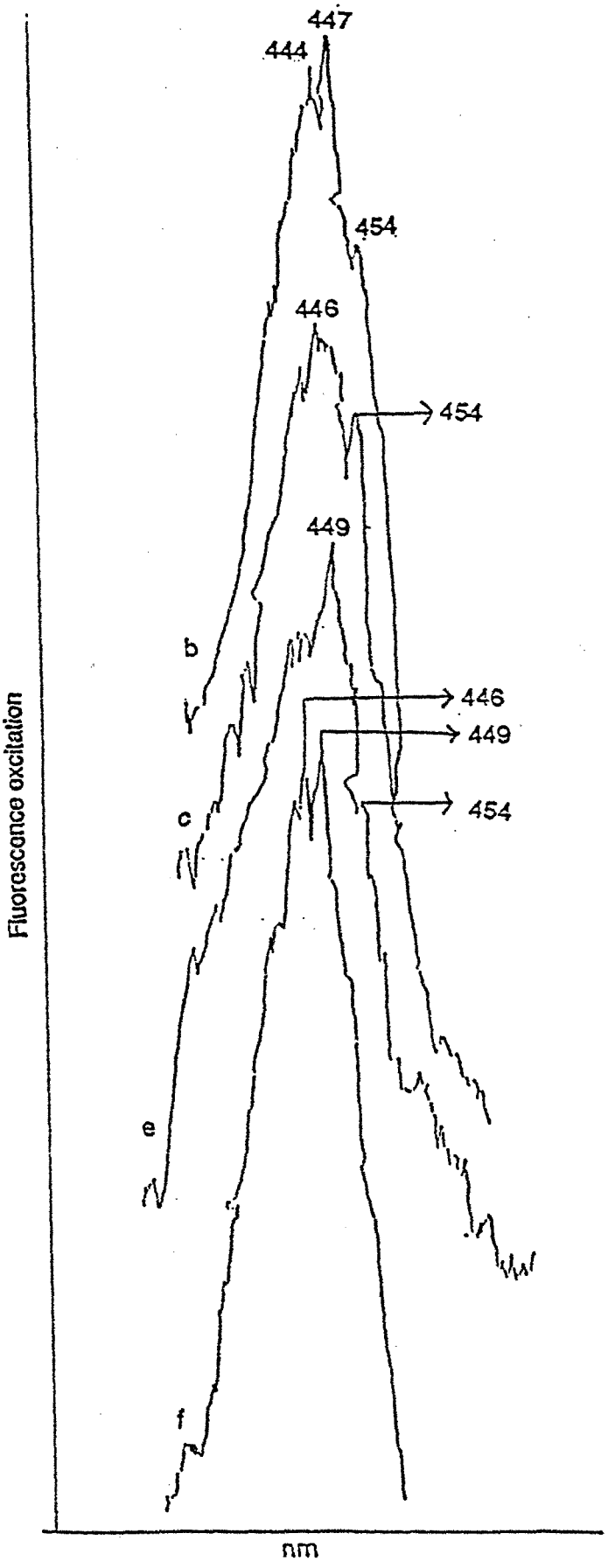

Fluorescence excitation
447
444
454
446
454
449
b
446
449
c
454
e
f
nm

Fig 3.7.2

The HPLC elution profile of the protochlorophyllide and chlorophyllide pools after various treatments described in Table 3.7.1. Samples were isolated from lysed cucumber etioplasts either (a) before, (b) immediately after, or (c-f) 10 min after the photoconversion. Sample contained either (b and c) no coenzyme, (d) NADH, (e) [4S-^{3}H]NADPH, or (f) [4R-^{3}H]NADPH.

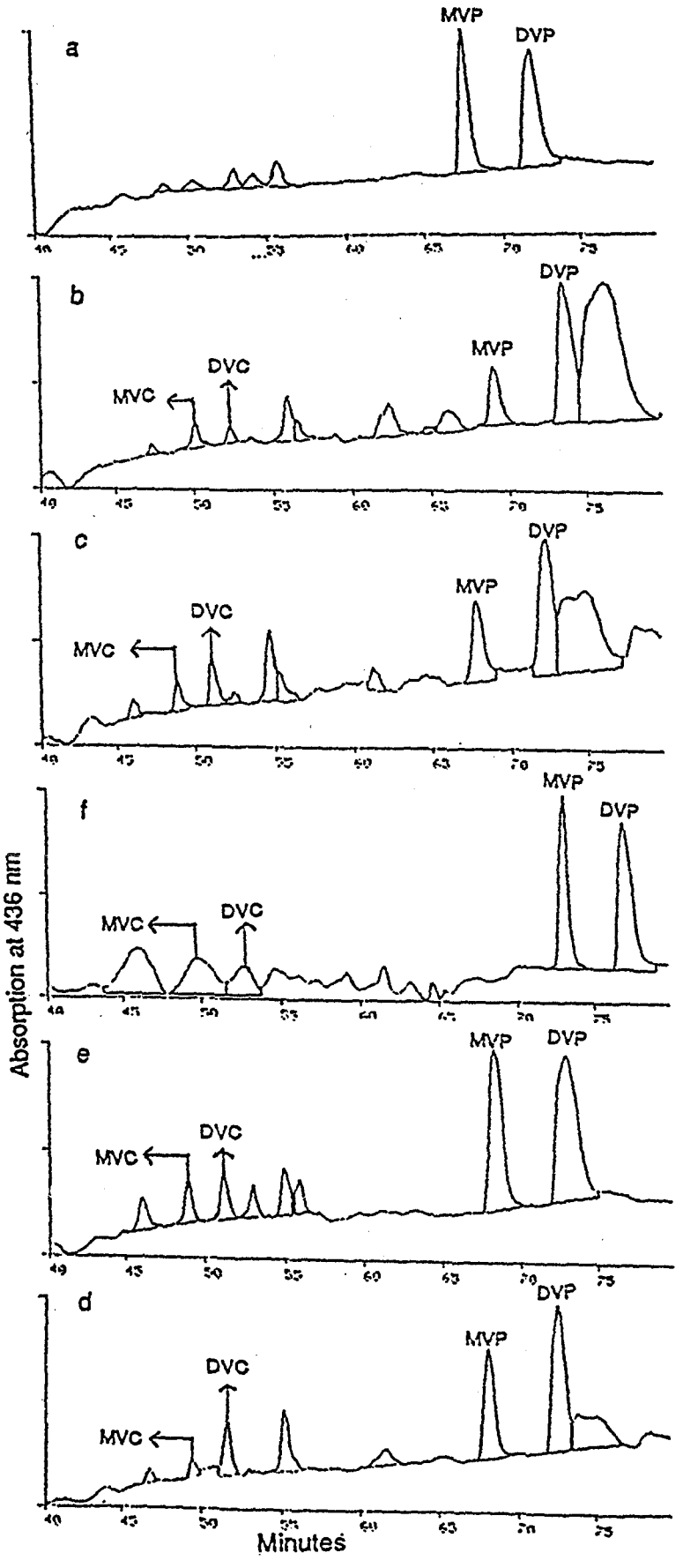

a
MVP
DVP
b
DVP
MVP
DVC
MVC
c
DVP
MVP
DVC
MVC
f
MVP
DVP
MVC
DVC
e
MVP
DVP
DVC
MVC
d
DVP
MVP
DVC
MVC
Absorption at 436 nm
Minutes

Fig 3.7.3

Tritium incorporation by the stereospecifically labeled coenzyme, [4R-^{3}H]NADPH, into pigments isolated from lysed cucumber etioplast membranes (o). The fractions, each containing 2.5 ml, were collected from an HPLC polyethylene column. The figure also shows the HPLC elution profile of sample (f) of Table 3.7.1 monitored at 436 nm. The absorption of the run from 20 to 80 min is expanded 3 times over the absorption of the run from 0 to 20 min.

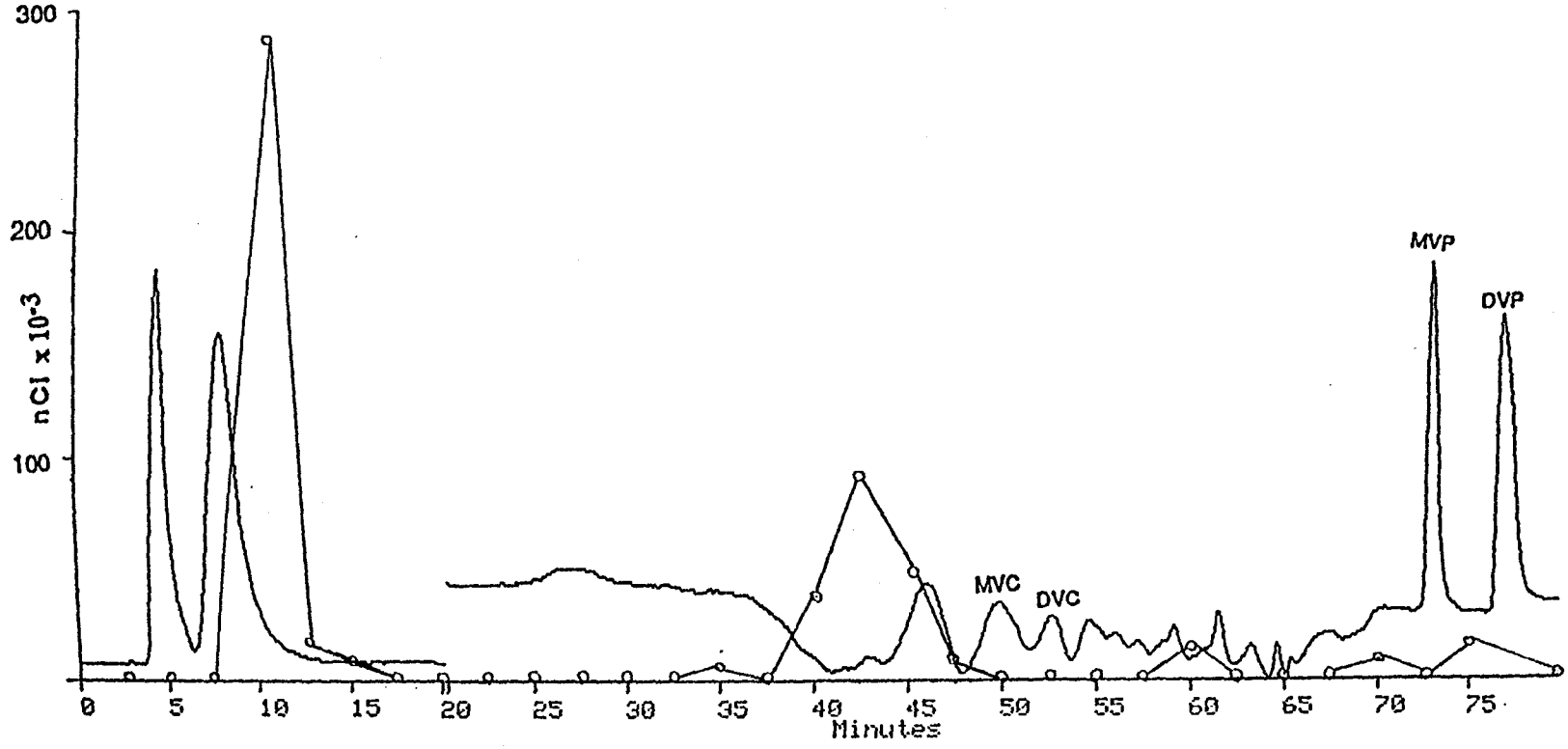
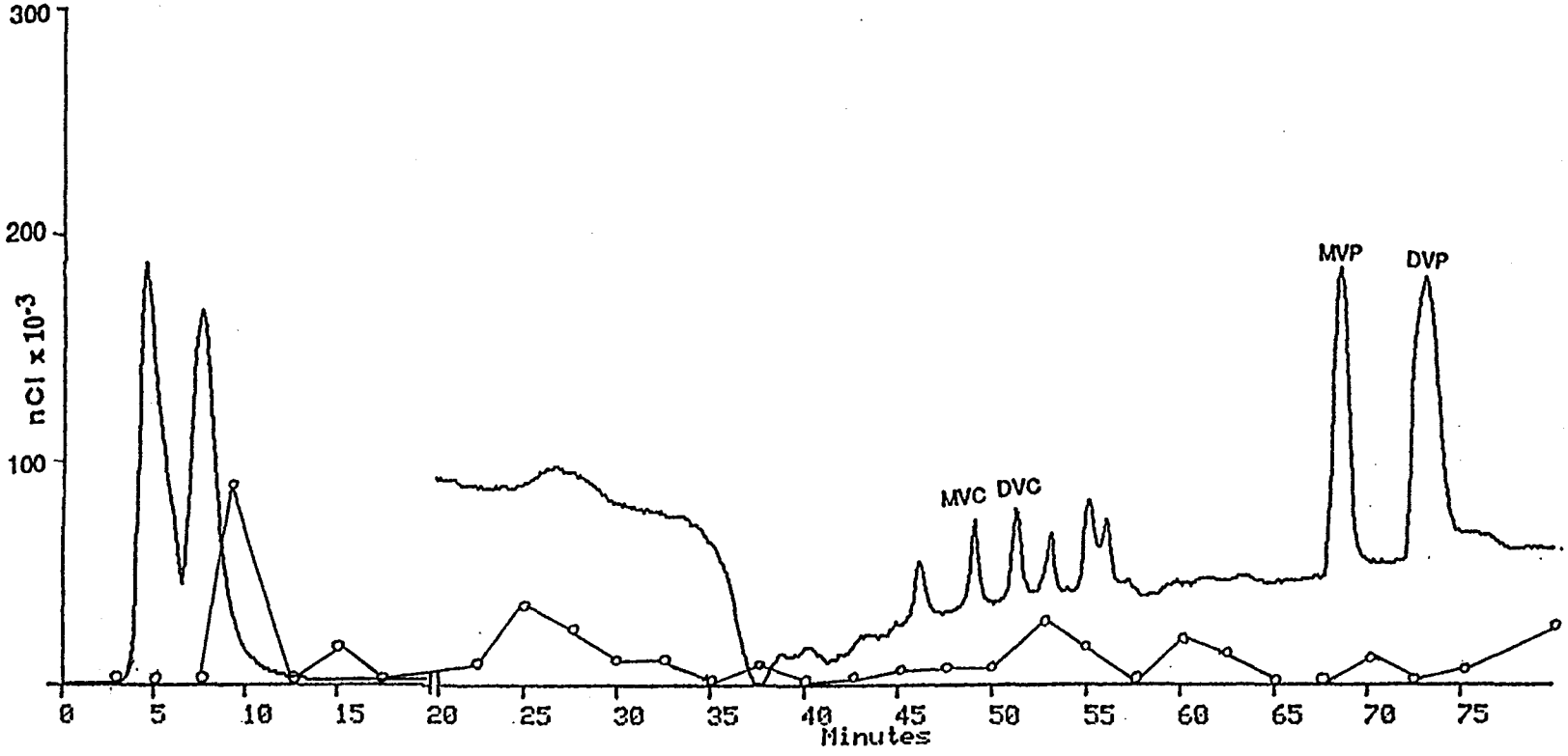

300
200
100
nCi x10-3
MVP
DVP
MVC
DVC
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Minutes

Fig 3.7.4

Tritium incorporation by the stereospecifically labeled coenzyme, [4S-^{3}H]NADPH, into pigments isolated from lysed cucumber etioplast membranes (o). The fractions, each containing 2.5 ml, were collected from an HPLC powdered polyethylene column. The figure also shows the HPLC elution profile of sample (e) of Table 3.7.1 monitored at 436 nm . The absorption of the run from 20 to 80 min is expanded 23 times over the absorption of the run from 0 to 20 min.


300
200
100
nCl x10-3
MVC
DVC
MVP
DVP
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Minutes

CHAPTER 4

DISCUSSION

The original scheme of the pathway of chlorophyll formation proposed by Granick (41) has been subjected to only minor modification (42,43). The aim of the work described in this thesis has been to demonstrate the formation of a monovinyl- from a divinyl tetrapyrrole in the biosynthetic pathway of chlorophyll *a*. This thesis also has been concerned with developing a reliable assay for the 4VR-enzyme in the formation of monovinyl tetrapyrroles.

A new HPLC solvent system for the separation of the chlorophyllide and protochlorophyllide pools using a polyethylene column was developed. This method, described in Chapter 2.3.2, allowed a reliable purification of MVC, DVC, MVP, DVP in a single run. When eluted, these compounds were characterized and confirmed by low temperature fluorescence spectroscopy as described in Chapter 3.6.

The discrepancies betwen HPLC and fluorescence assays has already been discussed in Chapter 3.6. All the results confirmed that the presence of either other tetrapyrroles or decomposition products shifts the wavelength higher or lower depending on the relative proportion of the compounds in the mixture.

The experiments described in Chapter 3.5 tried to determine one of the two potential substrates of 4VR, either DVP or DVC. The results confirmed that DVC is the 4VR substrate in whole cucumber etioplasts in the presence of endogenous coenzymes and also in lysed cucumber etioplast membranes in the presence of the exogenous coenzymes, NADPH and NADH. Unfortunately, we were unable to determine the 4VR substrate in wheat etioplasts and lysed wheat etioplast membranes. The reason was that the wheat PCR enzyme did not accept DVP as an exogenous substrate, and hence form DVC by light. Both

MVP and DVP are known substrates for the PCR enzyme of higher plants (44). All of our results have also indicated that both are substrates for the light-requiring enzyme, PCR.

Rebeiz et al. (45) gave evidence that not all higher plants become green the same way, since plants grown under field conditions may accumulate predominantly MVP or DVP either in darkness or in the light. Cucumbers are characterized as dark divinyl / light divinyl plants, and accumulate predominantly DVP under both light conditions. On the other hand, Walker et al. (46) had proposed that the reduction of the 4-vinyl group might take place somewhere between MgPME and DVP as the result of a study of the specifity of the oxidative cyclase enzyme system of cucumber towards synthetic MV- and DV-intermediates. It may be that the activity of the 4VR with such intermediates, or with DVP itself, may be too low to allow formation of MVP under steady-state conditions, and MVP is only formed under prolonged incubation in the dark. Reduction of the 4-vinyl group has now been shown in our laboratory to occur rapidly only between DVC and MVC in cucumbers. On the basis of our experiments, as a result of the action of light, all DVP in a photoconvertible PCR complex would be reduced to DVC. This then would dissociate from its PCR complex and be rapidly (within minutes) converted to MVC by the 4VR.

Chapter 3.7 described the dark incubation, carried out in broken cucumber etioplasts in the presence of either the 4R- or 4S- isomer of [^{3}H]NADPH. Our results showed that only in the case of the 4R-isomer was there any significant incorporation of radioactivity into the resulting tetrapyrrole fraction. However, there was no incorporation found in the expected product MVC, but radioactivity appeared within the unknown band name (referred to as UR with a maximum at 42.5 min) seen in Fig 3.7.3. The lack of incorporation into the expected product of the 4VR, MVC, can be explained by an indirect transfer of ^{3}H to the carbon-3

position of the pyrrole ring as proposed first by Michalski et al. (47). They suggested that the ethyl group at position 4 of Chl *a* and Bchl *a* could be formed from an ethylidine group (found naturally in Bchl *g*) following a shift of the proton at position 3. Walsh (48) described a number of well-characterized enzyme-catalyzed isomerizations of this kind (Fig 4.1). In such a case any tritium atom introduced from the nicotinamide ring into position 3 would be lost during a subsequent isomerization, yielding a 4- ethyl group (Fig 4.1). If this is the case, it would explain the observation that UR does not exhibit any absorption at 436 nm, which would support our suggestion that UR could have a similar structure to Bchl *g* (Fig 4.2) (Bchl *g* absorption is at 365, 405, 565, 766 nm (47)).

Michalski *et al.* (47) proposed that the ethylidine group (= $CH\text{-}CH_3$) is a normal intermediate in the biosynthesis of chlorophylls and that the $=CH\text{-}CH_3$ group is retained as such only by Bchl *g*, Bchl *b*, and by phycocyanobilin (41,49) and phycoerythrobilin (43). These are the chromophores of phycocyanin and phycoerythrin, respectively, the widely distributed auxiliary pigment-proteins in cyanobacteria. In all of these compounds, the $=CH\text{-}CH_3$ group is found in the same molecular position 4 of ring II. All that can be concluded, therefore, is that it appears likely that [4R-^{3}H]NADPH is used by cucumber 4VR to reduce DVC producing MVC, but the exact mechanism of the pro R-proton utilization has to be determined by future work. Several possible mechanisms can be hypothesized which imply the addition and subsequent loss of tritium. The most likely would be the mechanism proposed by Michalski *et al.* (47) (Fig 4.1).

Therefore, in order to determine the mechanism of the 4VR, the unknown tritium-labeled compound (UR) has to be purified and its structure determined. Future work must also require purification and determination of the absolute structures of all unknown bands (UP1, UP46, UP56, UP75); the compounds have to be purified and their structures analysed by NMR and mass

spectroscopy. B.Whyte (50) has already isolated an unknown band (named in this thesis UP1) and presented evidence that this compound is a degradation product.

In this work we were able to demonstrate the reduction of the 4-vinyl group of DVC but the requirement of the cofactors has yet to be still determined. The future work also requires:

1) The purification of 4VR.

Affinity chromatography has been used by Richards & coworkers to purify two enzymes [methyltransferase (MT) and PCR] of the magnesium branch of Chl *a* synthesis from etiolated wheat using metalloporphyrin substrate analogues as affinity ligands (14, 37, 51, 52). In the case of the 4VR, DVC would be the most likely affinity ligand to be used.

2) The determination of the physical properties of 4VR.

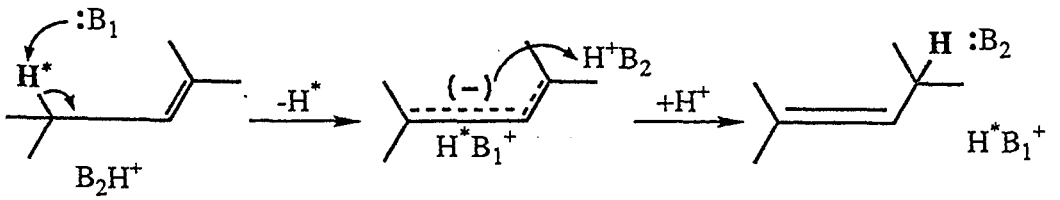
Once the 4VR has been successfully purified, the relative amount of the 4VR versus PCR (52) can be determined as well as the molecular weight of 4VR.

3) To determine the effect of light treatment of the plants on the 4VR.

One of the possible reasons for the presence of both MV- and DV-pathways in different plant species is differential light regulation on the production of 4VR isozymes which could be detected by isoelectric focussing of affinity chromatographically purified 4VR.

Fig 4.1

A. 1,3 proton shift during base-catalyzed isomerization.



B. The reduction of DVC by [4R-^{3}H]NADPH followed by base-catalyzed isomerization.

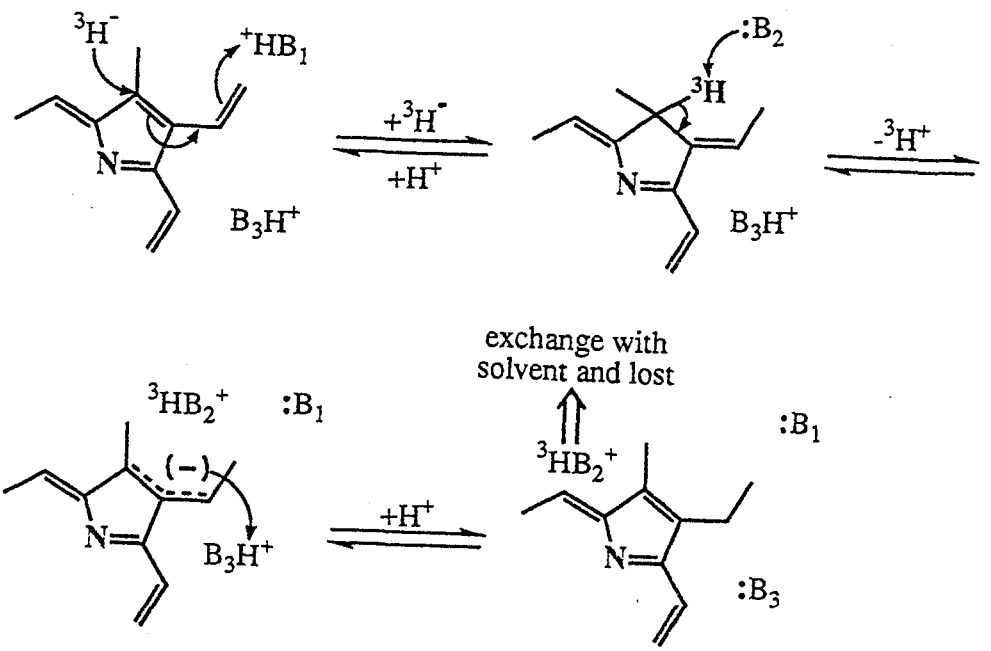
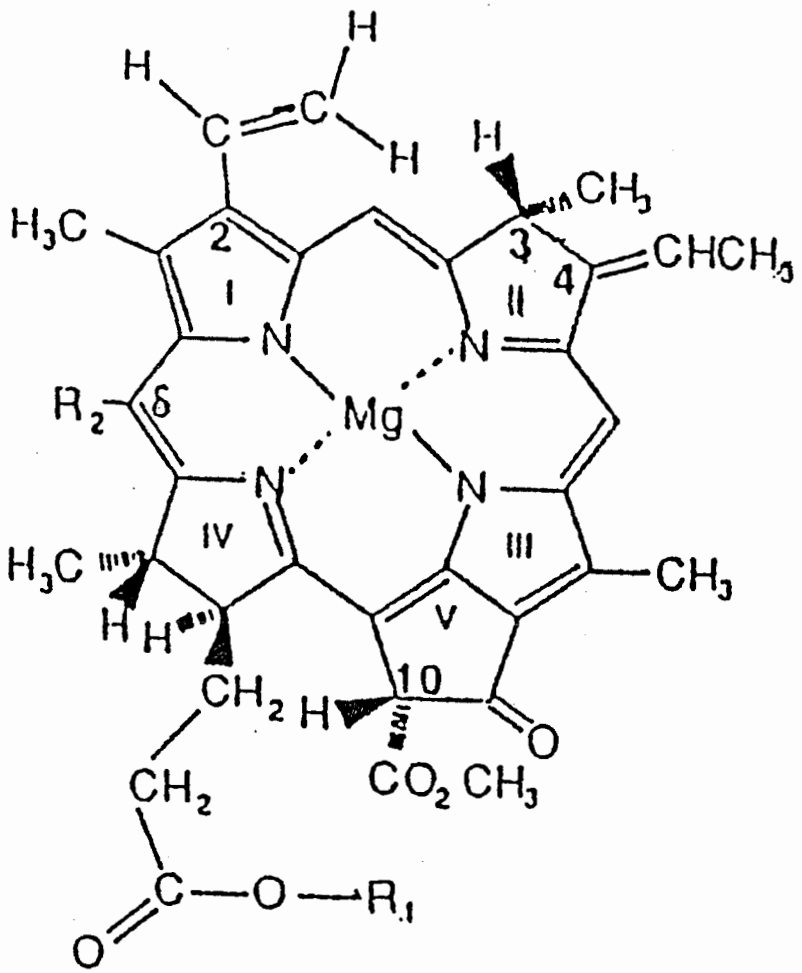


Fig 4.2

The molecular structure of bacteriochlorophyll *g* (R_1 = farnesyl).



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