THE SYNTHESIS OF MAGNESIUM AND ZINC PROTOPORPHYRIN IX AND THEIR MONOMETHYL ESTERS IN ETIOPLAST PREPARATIONS STUDIED BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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1. Introduction

The formation of the magnesium chelate of protoporphyrin IX is regarded as an essential step in the biosynthesis of chlorophyll from non-porphyrin precursors [1]. It is also one of the least understood steps in chlorophyll biosynthesis. This is partially due to the lack of suitable methods which permit the routine assay of the magnesium chelates of protoporphyrin IX and its methyl ester. Thus the formation of Mg-protoporphyrin IX with traces of its monomethyl ester was obtained on incubation of crude plastid preparations from greening cucumber cotyledons with L-glutamate as shown by TLC and fluorescence spectroscopy [2]. However, most of the experimental work on the properties of the synthesizing system utilized an acidic extraction procedure (which removed the central metal atom) and a spectrofluorimetric assay. The product was designated PROTO and included free protoporphyrin and metalloporphyrins which had lost their metal during the 'work up' procedure. In [3] Mg–protoporphyrin IX and the monomethyl ester in mixtures were directly assayed by spectrofluorimetry. This method failed to differentiate between Mg–protoporphyrin IX and 'related metalloporphyrins' [4] including Mg–protoporphyrin IX monomethyl ester. Further, cell-free preparations from etiolated wheat seedlings were unexpectedly shown to synthesize the zinc chelate of protoporphyrin IX rather than the magnesium chelate [5]. In view of this finding it is important that routine assays should be very specific and lead to the unequivocal identification of the reaction product.

We describe here an effective separation of the zinc and magnesium chelates of protoporphyrin IX, as the dimethyl ester derivatives, by HPLC. This separation has been applied in a routine manner to the examination of the protoporphyrin IX chelates arising from the incubation of cucumber etiochloroplast and wheat etioplast preparations with some non-porphyrin metabolic precursors of chlorophyll.

Abbreviations: ALA, 5-aminolaevulinic acid; BSA, bovine serum albumin; GSH, reduced glutathione; Hepes, 2-[*N*-2-hydroxyethylpiperazin-*N'*-yl] ethanesulphonic acid; HPLC, high pressure liquid chromatography; Mg–proto IX E, magnesium protoporphyrin IX monomethyl ester; proto IX, protoporphyrin IX; Tes, *N*-tris-[hydroxymethyl] methyl-2-aminoethanesulphonic acid; TLC, thin-layer chromatography

2. Materials and methods

2.1. *Chemicals*

Tes, GSH, BSA, $NADP^+$ and proto IX dimethyl ester were purchased from Sigma Chemical Co., St Louis, MO. Hepes was purchased from Calbiochem, San Diego, CA; L-glutamic acid from the British Drug Houses Ltd., Poole and ATP (disodium salt) from Boehringer Mannheim Australia, Melbourne. 5-Amino-[4-^{14}C]laevulinic acid hydrochloride (42 mCi/mmol) ([^{14}C]ALA), L[1-^{14}C]glutamic acid ([^{14}C]Glu) (50 mCi/mmol) and [*methyl*-^{14}C]toluene were supplied by the Radiochemical Centre, Amersham.

The dimethyl esters of Zn–proto IX and Mg–proto IX were synthesized for reference purposes from proto IX dimethyl ester using zinc acetate [6] and magnesium perchlorate [7]. Proto IX was prepared from its dimethyl ester by acid hydrolysis [6] and dissolved in 5% (v/v) ethanol in 0.01 M KOH for addition to reaction mixtures [3]. All other reagents were of analytical reagent grade.

2.2. *Plastid isolation and incubation*

Cucumber seeds (*Cucumis sativus* L. var. Supermarket) were germinated in the dark at ~25°C for 5.5–6 days in vermiculite moistened with tap water. Following greening for 6 h in light (incandescent tungsten, 70 μEinsteins $m^{-2}s^{-1}$) cotyledons were harvested and plastids isolated essentially as in [8]. Etiochloroplasts were isolated from 20–40 g tissue which was ground in a mortar and pestle in 60–120 ml isolation medium. The isolation medium contained sucrose (0.5 M), Tes (20 mM), Hepes (10 mM), $MgCl_2$ (1 mM), EDTA (1 mM), GSH (5 mM) and BSA (2 mg/ml), adjusted to pH 7.7 with KOH.

The plastid suspension (1–2 ml containing 6–13 mg protein/ml) was incubated for stated periods in white light (as for greening) at 27°C without stirring in the isolation medium supplemented with KH_2PO_4 (10 mM), ATP (1.5 mM) and $NADP^+$ (0.6 mM) at pH 7.7 and containing the appropriate substrate.

Seeds of wheat (*Triticum aestivum* (L.) Thell cv Mentana) were germinated in the dark at 25°C for 7–8 days in vermiculite moistened with distilled water. Etioplasts were released from enzymically prepared protoplasts as in [9]. Etiochloroplasts were prepared as for etioplasts except that the leaves were exposed to white light (as for cucumber cotyledons) for 3.5 h prior to excision and during protoplast isolation.

Etioplasts were incubated in a shaking water bath at 25°C for 6 h in the dark in 5 ml medium containing sorbitol (0.3 M), Hepes (50 mM), KH_2PO_4 (2 mM), GSH (5 mM) and $MgCl_2$ (0.75 mM) at pH 7.7 and containing 30 mg protein and 2.5 μCi [^{14}C]ALA. Etiochloroplasts (containing 27 mg protein) were incubated without shaking at 27°C for 2 h in the light in 2 ml of the same medium supplemented with NAD^+ (0.6 mM), EDTA (1 mM), ATP (2 mM) and 2 μCi [^{14}C]Glu.

Protein was determined using a variation of the Lowry method [10].

2.3. *Extraction of pigments into ether and separation by TLC*

Incubations were terminated and metalloporphyrins were extracted into diethyl ether as in [4]. A mixture of carrier pigments (see below) was added to those ether extracts obtained from incubations containing ^{14}C-labelled substrates. The ether extract was concentrated to 100–200 μl under N_2 and applied to a 20 × 20 cm Merck silica gel 60F254-coated glass thin-layer plate, cat. no. 5715, and developed in benzene:ethyl acetate:ethanol (8:2:2.5, by vol.). Substances of interest were detected on the chromatograms by their relative positions and characteristic fluorescence under long wavelength (~360 nm) UV light [11]. The bands were scraped off the plates either into vials for counting radioactivity or into tubes and the pigments extracted as follows. MPE was eluted from the silica by shaking with ~10 ml 80% aqueous acetone overlaid with 5–10 ml diethyl ether. If the two layers failed to separate immediately after shaking, they did so following the addition of 2–3 ml distilled water. The aqueous acetone containing the silica was re-extracted with additional ether (5–10 ml) until no fluorescence remained. This was usually achieved after 2 or 3 extractions. The combined ether fraction was washed twice with an equal volume of water and an aliquot placed in a scintillation vial if required to determine the amount of radioactivity present. Mg–proto IX was eluted from the silica as described for MPE except that a few crystals of KH_2PO_4 were added to the aqueous acetone fraction prior to extraction with ether.

2.4. *HPLC of metalloporphyrins*

The Mg–proto IX and Mg–proto IX E fractions from TLC in ether were esterified using diazomethane [6], concentrated to 2–3 ml under N_2, filtered through a millipore membrane (0.5 μm) and further reduced in volume under N_2 to 100–200 μl. HPLC was done at 15°C on a Brownlee S1-10A, 10 μm Li Chrosorb column using an Altex 110 pumping system equipped with an Altex 153 8 μl optical unit with a 405 nm interference filter. The solvent was acetone:hexane (15:85, v/v) and used at a 2 ml/min flowrate.

2.5. *Preparation of carrier pigments*

A mixture of carrier pigments (containing protochlorophyllide, Mg–proto IX E and trace amounts of Mg–proto IX and proto IX) in ether was obtained by incubating the top 10 cm of 7-day-old etiolated wheat leaves (~12 g) overnight in the dark at 25°C in 5 mM KH_2PO_4/KOH buffer (pH 6.9) containing ALA (5 mM) and 2,2-bipyridyl (10 mM). Following incubation, the leaves were homogenized in 80% aqueous acetone using an Ultra Turrax homogenizer and metalloporphyrins were extracted into diethyl ether as described for plastid metalloporphyrins. The metalloporphyrins in ether were concentrated under N_2 to ~20 ml and 100–200 μl (containing 1–3 nmol Mg–

proto IX E and 5–20 nmol protochlorophyllide) was added to those ether extracts obtained from plastids incubated with ^{14}C-labelled substrates. Mg–proto IX E isolated from this mixture and esterified using diazomethane, had chromatographic and spectral properties identical to those of standard Mg–proto IX dimethyl ester.

2.6. *Determination of radioactivity*

Methanol (0.5–1 ml) was added to the silica scrapings of pigments in scintillation vials followed by 10 ml scintillant (0.5% (w/v) 2,5-diphenyloxazole in toluene). Eluates from the TLC plates and from the HPLC column were evaporated to dryness under N_2 in scintillation vials and then 10 ml scintillant was added. Radioactivity was determined using a Packard tri-carb 2650 liquid scintillation counter. The efficiency of counting was determined using [^{14}C]toluene as an internal standard.

2.7. *Atomic absorption spectrometry*

HCl (1 M) extracts were prepared from metalloporphyrins in ether as in [12]. Magnesium and zinc were determined in these extracts using a Varian Techtron model 80 atomic absorption spectrometer.

Absorption spectrophotometry was performed on a Cary 118C UV–visible spectrophotometer.

3. Results

3.1. *HPLC of metalloporphyrins*

HPLC effectively separated the dimethyl esters of Zn–proto IX and Mg–proto IX (fig.1). There appeared to be no overlap. In this system proto IX dimethyl ester has a retention time of 5 min.

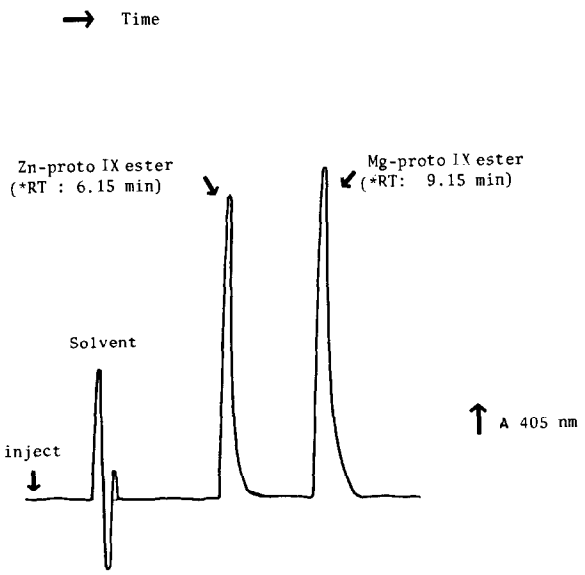

Fig.1. Separation of a mixture of Mg–proto IX dimethyl ester and Zn–proto IX dimethyl ester standards by HPLC. 10 μl of a mixture containing ~80 μmol/ml of each standard pigment was subjected to HPLC as described in section 2. *RT, retention time

3.2. *Metalloporphyrin formation from [^{14}C]glutamate or [^{14}C]ALA*

Cucumber etiochloroplasts incubated with either [^{14}C]Glu or [^{14}C]ALA accumulated radioactivity in both the Mg–proto IX and the Mg–proto IX E fractions from TLC (table 1). Amounts of radioactivity similar to those observed for Mg–proto IX were found to accumulate in chlorophyll also (not shown). The calculated values for metalloporphyrin formation would be lower than actual since the calculation assumes no endogenous glutamate or ALA in the plas-

Table 1
The accumulation of radioactivity in Mg–proto IX by cucumber etiochloroplasts incubated with ^{14}C-labelled substrates

Expt	Substrate	[Plastid] (mg protein/ml)	Mg–Proto IX		Mg–proto IX E	
			dpm	pmol . h^{-1} . mg protein^{-1}	dpm	pmol . h^{-1} . mg protein^{-1}
1	[1-^{14}C]Glu	9.05	11 841	0.96	2415	0.20
2	[4-^{14}C]ALA	9.70	228 423	15.79	6193	0.43
3	[1-^{14}C]Glu	9.15	7778	0.48	1366	0.08
	[4-^{14}C]ALA		74 316	5.44	2898	0.21

Plastid suspensions were incubated with 1 μCi/ml [^{14}C]ALA or [^{14}C]glutamate and ^{14}C-labelled metalloporphyrins isolated as TLC fractions as in section 2

Table 2
High pressure liquid chromatography of ^{14}C-labelled metalloporphyrins

Plastids	Substrate	Zn–proto IX (dpm)	Mg–proto IX (dpm)
A.			
Cucumber	(a) [1-^{14}C]Glu (2 μCi/ml)	105	2364
Etiochloroplasts	(b) [4-^{14}C]ALA (1 μCi/ml)	220	16 420
B.			
Wheat etioplasts	[4-^{14}C]ALA (0.5 μCi/ml)	13 530	200

(A) Cucumber etiochloroplasts were incubated with ^{14}C-labelled substrates as follows: (a) 1 ml plastid suspension containing 11.8 mg protein was incubated for 1.5 h; and (b) 1 ml plastids containing 9.2 mg protein was incubated for 2 h. Metalloporphyrins were separated by TLC and the Mg–proto IX and Mg–proto IX E fractions from TLC combined, methyl-esterified using diazomethane and concentrated under N_2. ~100 nmol standard Zn–proto IX dimethyl ester were added to each extract and ~1/3 of each was subjected to HPLC

(B) Wheat etioplasts were incubated and metalloporphyrins isolated by TLC as in section 2. The Mg–proto IX E fraction only was eluted, esterified and a portion (~2/3) subjected to HPLC in the presence of Zn–proto IX dimethyl ester

The two fractions containing the metalloporphyrins were collected, dried under N_2 and the radioactivity present in each was determined

tid preparation. In addition it is not surprising that [^{14}C]ALA as substrate results in the accumulation of more metalloporphyrin than [^{14}C]Glu since the pathway from glutamate to ALA is relatively unstable in vitro [13,14].

The Mg–proto IX and MPE fractions from TLC from such incubations were combined, esterified by means of diazomethane, supplemented with Zn–proto IX dimethyl ester and subjected to HPLC. As shown in table 2 the magnesium chelate retained the radioactivity whereas practically none appeared in the zinc chelate.

Wheat etioplasts incubated in the dark with [^{14}C]-ALA also accumulated radioactivity in the TLC region corresponding to Mg–proto IX E. In contrast to the cucumber preparations however, the radioactivity accompanied the zinc derivative during HPLC (table 2). Wheat etiochloroplasts incubated in the light with [^{14}C]Glu (see section 2) failed to accumulate significant amounts of radioactivity in any porphyrin fraction.

3.3. *Mg–proto IX synthesis by cucumber etiochloroplasts incubated with 5 mM L-glutamate*

Relatively large amounts of Mg–proto IX [3–18 pmol h^{-1} (mg plastid $protein^{-1}$)] were accumulated by cucumber etiochloroplasts incubated with 5 mM L-glutamate [3]. The synthesis of this pigment was stimulated by as much as 10-fold by the inclusion of exogenous proto IX (5 μM) in the reaction mixture.

The ability of our cucumber preparations to synthesize Mg–proto IX from L-glutamate and proto IX was examined to ascertain whether enough of the Mg–proto IX fraction would accumulate to enable more definitive analytical data to be obtained which would confirm that it was indeed the magnesium chelate of proto IX. After incubation of etiochloroplasts as in treatment (c) of table 3, the major products discernable after TLC were proto IX; the Mg–proto IX fraction and an unidentified pigment which may be similar to the degradation product in [15]. On esterifying the Mg–proto IX fraction, it was subjected to HPLC whereby it chromatographed with a retention time corresponding to that of standard Mg–proto IX dimethyl ester, and well removed from that of the zinc derivative. HPLC effectively removed a small amount of contaminant (the unidentified pigment) and quantitative spectrophotometric determination was possible. The presence of 5 μM proto IX in the incubation mixture in addition to 5 mM L-glutamate brought about a 4-fold increase in Mg–proto IX accumulation [table 3, treatment (d)]. Small amounts of MPE were also apparent in extracts from plastids incubated with both glutamate and proto IX, however, the levels of this pigment were insufficient to permit quantitation by absorption spectrophotometry.

Mg–proto IX obtained under the conditions of

Table 3
Mg–proto IX synthesis in cucumber etiochloroplasts incubated with non-radioactive substrates

Treatment	Mg–proto IX (pmol . h^{-1} . mg $protein^{-1}$)
(a) Zero time	n.d.
(b) No additions	n.d.
(c) + 5 mM L-glutamate	1.8
(d) + 5 mM L-glutamate + 5 μM proto IX	7.5

Cucumber plastids were incubated for 2 h and a number of incubation mixtures were pooled prior to extraction of metalloporphyrins into ether and TLC. The TLC band corresponding to Mg–proto IX was eluted into diethyl ether, methyl esterified and subjected quantitatively to HPLC. Each treatment was comprised of 6.5 ml total vol containing av. 12.6 mg protein/ml. Mg–proto IX was determined in the HPLC solvent using ϵ_{589} 1.67×10^4 M^{-1} . cm^{-1} which was derived by comparison with its A_{589} in diethyl ether [16]. n.d., none detected

table 3 treatment (d) was used in the analyses reported in tables 4,5. These results clearly identify the pigment synthesized from L-glutamate and proto IX as the magnesium chelate of proto IX. The zinc chelate of proto IX was not detected in any of the extracts examined.

4. Discussion

Isolated wheat etioplasts incubated with [^{14}C]-ALA formed the zinc chelate of proto IX rather than the magnesium chelate in [5]. That observation is confirmed here. It is clear that for studies in vitro, adequate identification of reaction products should be made. In [5] a TLC system was used to separate the magnesium and zinc chelates of proto IX dimethyl ester. Here, an HPLC technique giving a very clean separation of the two chelates and suitable for routine quantitation of Mg–proto IX chelatase activity in cell-free extracts, is described. It is possible to omit the TLC step, treat the ether extract of incubation mixtures with diazomethane and then subject it directly to HPLC. The Mg–proto IX dimethyl ester separates from other porphyrins and can be used without further purification to study in a quantitative manner the formation of Mg–proto IX from [^{14}C]-ALA and [^{14}C]Glu.

By means of the HPLC separation it has been shown

Table 5
Determination of magnesium and zinc in the metalloporphyrin isolated from cucumber etiochloroplasts

Metalloporphyrin (nmol)	Mg (ng atoms)	Zn (ng atoms)
15.5	18.1	0

The metalloporphyrin in table 4 was dried under N_2, and treated with 1 M HCl as in section 2. Magnesium and zinc were determined in the extract by atomic absorption spectrometry. The amount of metalloporphyrin in the sample was determined from its A_{589} in diethyl ether [16]

Table 4
Visible light absorption characteristics of the metalloporphyrin isolated from cucumber etiochloroplasts incubated with 5 mM L-glutamate and 5 μM proto IX

	Soret	λ_{max} (nm) β	α
Mg–proto IX dimethyl ester standard	417	549–550	589–590
R	18.0	1.04	1.00
Zn–proto IX dimethyl ester standard	414	542–543	580–581
R	13.9	0.88	1.00
Esterified metalloporphyrin isolated from reaction mixtures	417	550	589
R	17.5	1.04	1.00

The metalloporphyrin from several incubations was purified by TLC and HPLC as in the legend to table 3. The pigment was collected from the HPLC column, the solvent evaporated under N_2 and the metalloporphyrin dissolved in diethyl ether for spectrophotometry. R, relative peak height

that cucumber etiochloroplasts, in contrast to wheat plastids, accumulate the magnesium chelates of proto IX and its monomethyl ester when either [^{14}C]ALA, [^{14}C]Glu or 5 mM glutamate was substrate. The nature of the accumulated material was positively identified from HPLC retention times, visible absorption spectrophotometry and atomic absorption spectrophotometry. This supplements the data in [2,3] and, using new specific analytical data, verifies the conclusion that preparations of cucumber etiochloroplasts actively insert magnesium into proto IX.

Wheat etioplasts incubated with [^{14}C]ALA were shown to accumulate a pigment with chromatographic properties identical to those of ^{14}C-labelled Zn–proto IX monomethyl ester. A preliminary report of this work [16] described the effect of osmotically rupturing the plastids. Following rupture, incorporation of [^{14}C]ALA into free porphyrins and Zn–proto IX monomethyl ester increased 10-fold suggesting that the etioplast envelope restricted the entry of ALA into the stroma region of the plastids. The factors effecting the formation of the Zn chelates of protoporphyrin IX and its ester rather than the Mg chelates in these wheat etioplast preparations are not understood.

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