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The assay of chlorophylls *a* and *b* converted to their respective magnesium-rhodochlorin derivatives by extraction from recalcitrant algal cells with aqueous alkaline methanol: prevention of allomerization with reductants

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A new method is described for assaying the difficult-to-extract (refractory) chlorophylls from so-called recalcitrant algal cells by extraction at 60 °C with 2% KOH in aqueous methanol containing 1.5 mM sodium dithionite and 15% (v/v) water. Chlorophylls *a* and *b* were converted during this extraction, by hydrolytic cleavage of both the phytyl ester bond and of the *iso*cyclic ring, to magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester and its 7^1-oxo derivative, referred to as magnesium-rhodochlorins *a* and *b*: the chlorophylls were assayed as these derivatives. The alkaline conditions may also hydrolyze the cell walls or chloroplast membranes to make these structures permeable to the extractant. Competing allomerization reactions (i.e., the oxygen-mediated cleavage of the *iso*cyclic ring), which so readily occurs when algal or plant material is treated at room temperature with alkaline methanol, were avoided by the addition of reductants such as dithionite, ascorbate, dithiothreitol or mercaptoethanol; however, dithionite was preferred. When determining the extinction coefficients of magnesium-rhodochlorins *a* and *b*, it was found that no allomerization occurred when purified chlorophylls *a* and *b* were converted aerobically in alkaline methanol at room temperature, even in the absence of reductants. This suggests that plant and algal extracts contain allomerization-enhancing compounds. The complete extraction of refractory chlorophylls from the recalcitrant green alga, *Nanochloris atomus* (strain CS 183), with 2% KOH in 85% aqueous methanol containing 1.5 mM dithionite at 60 °C is reported here: only 74 and 7% of the chlorophylls were extracted with methanol and buffered 80% aqueous acetone, respectively. The presence of 15% water in the alkaline methanol was also essential for total chlorophyll extraction from this green alga, but the reductant was not which indicates that heating to 60 °C helps to diminish allomerization by removing dissolved oxygen from the solvents; nonetheless, reductant was still added to ensure that no allomerization occurs during manipulations prior to heating. This new procedure is recommended to replace the older alkaline pyridine extraction method for refractory chlorophylls (see Porra, R.J. and Grimme, L.H. (1974) Anal. Biochem. 57, 255–267) because the cyclic hydroxylactone derivatives of chlorophylls *a* and *b*, formed in alkaline pyridine, are less stable than magnesium-rhodochlorins *a* and *b*. Further, alkaline methanol has the advantage that it is less pungent than alkaline pyridine.

Introduction

For many decades it has been known that it is very difficult to extract chlorophylls from some fresh-water and marine micro-algae using the extractants normally employed for higher-plant chlorophylls, but the underlying cause is not clear. Possibly, the chlorophylls are 'refractory' to extraction because of the nature of their bonding in the thylakoid-membrane-spanning proteins or the immediate molecular environment of the chlorophyll complexes in the thylakoid membrane. Alternatively, the difficulty of extraction may be due to some property of the 'recalcitrant' cell, such as an impermeable cell wall or chloroplast membrane which resists penetration by the solvent. Supporting this latter view are the armoured dinoflagellates with their cellulose

Abbreviations: Chl, chlorophyll; DMF, *N*,*N*′-dimethylformamide; Mg-Rchln *a*, magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester; Mg-Rchln *b*, magnesium-3^1,3^2-didehydro-7^1-oxorhodochlorin-15-acetic acid 13,15,17-trimethyl ester.

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walls, heavily silicified berthic diatoms, blue-green algae with their multi-layered walls and heavily-walled green algae which often do not release their pigments to aqueous acetone or methanol: much stronger solvents such as dimethylsulphoxide, freezing and thawing techniques and mechanical grinding of frozen cells on filters are frequently employed, but often without achieving total extraction (Jeffrey, S.W., personal communication).

In 1974, Porra and Grimme [1] extracted refractory Chls *a* and *b* from cells of regreening cultures of *Chlorella fusca* [2] with 2.1 M pyridine in 0.35 M NaOH at 60 °C. In the alkaline pyridine reagent the phytyl ester linkage was hydrolyzed and, in addition, the *iso*-cyclic ring was cleaved by oxygen-mediated allomerization reactions to stoichiometrically convert the chlorophylls to their cyclic hydroxylactones (see Fig. 6; Structure V). In this paper, refractory chlorophylls were extracted using alkaline methanol which again dephytylated the chromophore but the *iso*cyclic ring was cleaved by hydrolysis rather than by an oxidative allomerization reaction and the pungent pyridine was replaced with methanol. The conversion of purified Chls *a* and *b* in alkaline methanol to magnesium-3^1,3^2-didehydrorhodochlorin-15-acetic acid 13,15,17-trimethyl ester and magnesium-3-didehydro-7-oxorhodochlorin-15-acetic acid 13,15,17-trimethyl ester, respectively (see Fig. 6; Structure I), by non-oxidative solvolysis [3] was earlier demonstrated by a number of workers [4–7]. The nomenclature and numbering system used here is that currently approved by the International Union of Biochemistry [8,9] and for brevity and convenience these two derivatives from Chls *a* and *b* are referred to, respectively, as magnesium-rhodochlorins *a* and *b* (i.e., Mg-Rchlns *a* and *b*); these two compounds formerly enjoyed the trivial names, Mg-chlorin e_6 and Mg-rhodin g_7, respectively [10].

Alkaline methanol was used previously by Milner et al. [11] to extract chlorophylls from plant chloroplasts. They devised an assay, using a simple photoelectric colorimeter, for total chlorophylls but the experiments reported here show that the products were not the 'saponified chlorophylls' anticipated by these authors, but were Rchlns *a* and *b*. Although Milner et al. [11] did not attempt to determine individual concentrations of Chls *a* and *b*, the major red peaks of Mg-Rchlns *a* and *b* (like those of the chlorophylls from which they are derived) occur approx. 18 nm apart but at 642 and 624 nm [7], respectively; thus, determination of the extinction coefficients of both Mg-Rchlns at the above wavelengths allowed the derivation of simultaneous equations to calculate the individual concentrations of the original Chls *a* and *b* from which they were derived.

This paper also shows that dithionite inhibited allomerization reactions, which occur (especially at room temperature) during the treatment of plant and algal cells with alkaline methanol, so that Chls *a* and *b* in the cells were converted stoichiometrically to Mg-Rchlns *a* and *b*.

Finally, Chls *a* and *b* are shown to be totally extracted from cells of the recalcitrant green algae, *Nannochloris atomus* (strain CS 183), with 2% KOH in 85% aqueous methanol containing 1.5 mM dithionite: the presence of about 15% H_2O in the extractant was found to be essential to the removal of chlorophylls from recalcitrant cells.

Experimental

Chemicals and reagents. Acetone, diethyl ether and methanol were all AnalaR grade reagents supplied by BDH Chemicals (Australia), Port Fairy, Australia. DMF was supplied by May & Baker, West Footscray, Australia. Dithionite (analytical grade) was obtained from E. Merck, Darmstadt and dithiothreitol from Boehringer-Mannheim. Sodium ascorbate was obtained from Sigma. Buffered 80% aqueous acetone is 80% aqueous acetone containing either 2.5 mM sodium phosphate buffer (pH 7.8) or 50 mM Hepes-KOH buffer (pH 7.5) to minimize conversion of chlorophylls to phaeophytins.

Alkaline methanol reagent was prepared by the addition, just before use, of either 1.50 mM sodium dithionite, 2.0 mM sodium ascorbate or 1.25 mM dithiothreitol to 2% KOH in methanol. Aqueous alkaline methanol reagent, for use with cells containing refractory chlorophylls, in addition contained 15% water: the methanol for both reagents and the water were freshly boiled to reduce the concentration of dissolved oxygen. See the Discussion section for considerations affecting the choice of the most appropriate reductant.

Purification of chlorophylls a and b. Chls *a* and *b* were purified by a modification of the method of Porra et al. [7]. Maize leaves were extracted with buffered 80% aqueous acetone and the chlorophylls were precipitated with dioxane. The precipitated chlorophylls were redissolved in acetone and transferred to petroleum ether (40–60 °C) by dilution with 10% aqueous NaCl prior to application to a sucrose column. Chls *a* and *b* were separated on the column with 0.5% *n*-propanol in petroleum ether. Each chlorophyll was removed from the sucrose with diethyl ether and the solvent was removed by evaporation under reduced pressure. Each chlorophyll was then redissolved in acetone and transferred to petroleum ether and rechromatographed as described above. The presence of C-13^2-hydroxy- or -methoxychlorophylls in the diethyl ether solution of the rechromatographed samples of Chls *a* and *b* was checked by HPTLC [7]. Because the hydroxy- or methoxy-Chls *a* and *b* are not hydrolysed to Rchlns *a* and *b* in alkaline methanol, their presence can also be detected by the presence of residual 'chlorophyll' peaks or shoulders at

approx. 665 and 652 nm, respectively, when Mg-Rchln *a* and *b* formation is complete.

Determination of extinction coefficients of Mg-Rchlns a and b in alkaline methanol reagent. To avoid crystallizing, drying and weighing readily oxidizable Chls *a* and *b*, we used solutions of these highly-purified chlorophylls in diethyl ether as our primary standards. The concentrations of these standards were calculated using the extinction coefficients of Smith and Benitez [12] which have been verified by magnesium determination using atomic absorption spectroscopy [7]. The specific extinction coefficients (α) for Chls *a* and *b* in diethyl ether are 100.9 and 62.0 $l \cdot g^{-1} \cdot cm^{-1}$, respectively, at 660.8 and 642.5 nm [12]: the corresponding millimolar extinction coefficients (ϵ_{mM}) in diethyl ether are 90.2 and 56.3 $l \cdot mmol^{-1} \cdot cm^{-1}$, respectively [7].

The extinction coefficients of Mg-Rchlns *a* and *b* in alkaline methanol reagent were obtained by comparing the spectra of Chls *a* and *b* in diethyl ether with the spectra of the corresponding Mg-Rchln derivatives in an equimolar solution in the alkaline methanol reagent. This was achieved using a 5 ml graduated cuvette fitted with a B-14 stopper secured with two extension springs to minimize errors due to evaporation of volatile solvents [7]. Precautions, previously described [7], were taken against both variable micro-contamination and against contamination by oxidation and demetallation products during preparation and spectrophotometric measurement of the Mg-Rchln derivatives.

The spectra of Chls *a* or *b* in 5 ml of diethyl ether, automatically zeroed at 750 nm, were recorded between 750 and 550 nm in a stoppered 5 ml graduated cuvette. The cuvette was opened and the diethyl ether, which was removed by evaporation under a stream of oxygen-free nitrogen, was replaced with an equal volume of methanol; the cuvette was closed and the spectrum was recorded. The cuvette was again opened and dithiothreitol (1.0 mg) was added and dissolved, followed by 100 mg of powdered KOH: the cuvette was quickly closed and secured before careful inversion several times to dissolve the alkali. This procedure was always used when dissolving or extracting chlorophylls with alkaline methanol reagent or aqueous alkaline methanol reagent to ensure the presence of reductant (dithiothreitol or dithionite) before exposure to alkaline conditions. Spectra from 750 to 550 nm, and automatically zeroed at 750 nm, were recorded at room temperature at specified intervals as the reaction proceeded to completion. Room temperature slowed the reaction sufficiently (100 min for Chl *a* and 20 min for Chl *b*) to allow a number of spectra to be recorded before its completion: automatic zeroing at 750 nm enabled the observation of isosbestic points.

The same procedure was followed when using aqueous alkaline methanol reagent except that the chlorophylls were redissolved in methanol (4 ml) before adding H_2O (0.75 ml) and adjusting to 5 ml with methanol. When pure chlorophylls were treated with either alkaline methanol reagent, the reactions could be speeded up by incubating at 60 °C: only 20 min was required for the reaction of a mixture of both chlorophylls to proceed to completion.

Extraction of chlorophylls from leaf discs with alkaline methanol reagent. Leaf discs were ground in alkaline methanol reagent (2 ml) with pestle and mortar, and the mortar was rinsed three times with 1.5 ml of the reagent: as this extraction was performed at room temperature, reductant was added to prevent allomerization. The pooled homogenate and washings were transferred to a stoppered centrifuge tube and centrifuged. After centrifugation, the pellet was extracted once or twice more with 2 ml of reagent until the pellet was no longer green or exhibiting red fluorescence under ultraviolet light. The final volume of the combined supernatants was noted and after 100 min at room temperature to permit complete conversion to Rchlns *a* and *b*, spectra were recorded between 750 and 550 nm.

Extraction of chlorophylls from cells of N. atomus (strain CS 183). These algal cells, generously provided by Dr. Shirley Jeffrey of the CSIRO-Division of Fisheries Research, Hobart, were harvested by centrifugation and then suspended, using a Potter-Elvejhem homogenizor, in aqueous alkaline methanol reagent (5 ml) containing dithionite: the KOH was added last as a fine powder as described above. This suspension was transferred to a screw-capped centrifuge tube, closed and wrapped in foil to exclude light, and heated at 60 °C for 20 min. After centrifugation, the pellet was extracted in the same manner with a further 5 ml of reagent until the pellet was no longer green or exhibiting red fluorescence under ultraviolet light: normally, only three extractions were needed. The final volume of the pooled supernatants was noted before spectrophotometric assay.

Spectra. All spectra and spectrophotometric measurements were obtained using a Hitachi Model U3200 Recording Spectrophotometer.

Results

Determination of the extinction coefficients of Mg-Rchlns a and b and the derivation of simultaneous equations for their assay

Initial studies of the reaction of pure Chls *a* and *b* with 2% KOH in methanol at room temperature showed that the *iso*cyclic rings were opened by simple hydrolysis without any oxygen-mediated cleavage of these rings (i.e., allomerization) even in the absence of reductant: the spectra of the Mg-Rchlns *a* and *b* formed are shown in Figs. 1 and 2. Further proof of the identity of these Mg-Rchlns was obtained by preparing the magnesium-free derivatives by aqueous dilution of the alkaline methanol solutions prior to adjusting to pH 2

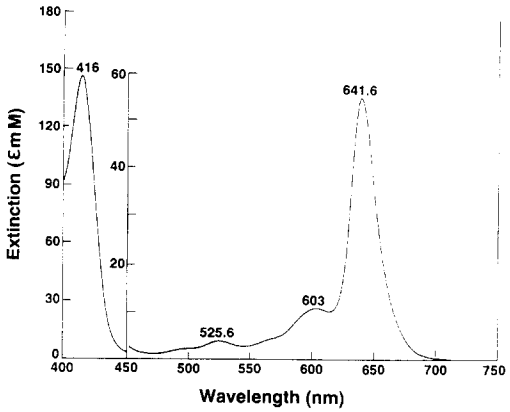

Fig. 1. Spectrum of Mg-Rchln *a*. A solution of pure Chl *a* in diethylether (5 ml) was evaporated to dryness under O_2-free N_2 at 60 ° C and then redissolved in 5 ml of 2% KOH in methanol in a 5 ml stoppered cuvette. This spectrum was recorded when the reaction was complete.

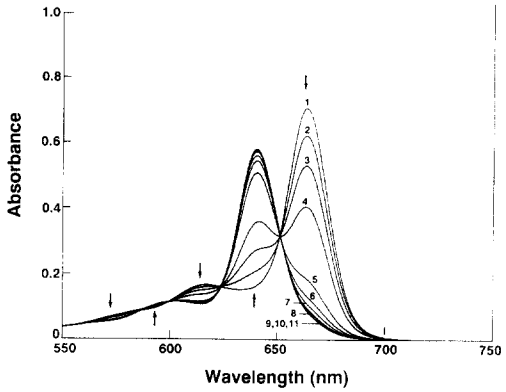

Fig. 3. Spectra of the reaction mixture during the conversion of pure Chl *a* to Mg-Rchn *a* in alkaline methanol reagent (containing 1.25 mM dithiothreitol) at room temperature. Spectrum 1 (zero time) was recorded in methanol. Spectra 2-11 were recorded at 1, 3, 8, 20, 35, 45, 55, 70, 80 and 100 min after addition of solid dithiothreitol followed by KOH (100 mg) Spectra 9, 10 and 11 are indistinguishable. Isosbestic points can be clearly seen.

and extracting into diethyl ether: the spectra of these ethereal solutions (not shown) were identical to those of Rchln *a* and *b* reported by Porra [13], referring to them by their previous names, chlorin e_6 and rhodin g_7, respectively.

The spectra in Figs. 3 and 4 record the conversion of Chls *a* and *b*, respectively, to Mg-Rchlns *a* and *b* at room temperature in alkaline methanol reagent containing 1.25 mM dithiothreitol. Spectrum 1 in both Figs. 3 and 4 is of the parent chlorophyll in methanol (5 ml) after the removal of diethyl ether and prior to the addition of solid dithiothreitol and KOH (100 mg) (see Experimental). The three identical spectra, 9, 10, and 11, of Fig. 3 taken at 70, 80 and 100 min, indicating that the reaction is complete, represent the spectrum of Mg-Rchln *a*. Similarly, identical duplicate spectra 6 and 7 of Fig. 4, taken at 15 and 20 min, represent the spectrum of Mg-Rchln *b*. Clearly, the reaction is more rapid with Chl *b* than with Chl *a* at room temperature.

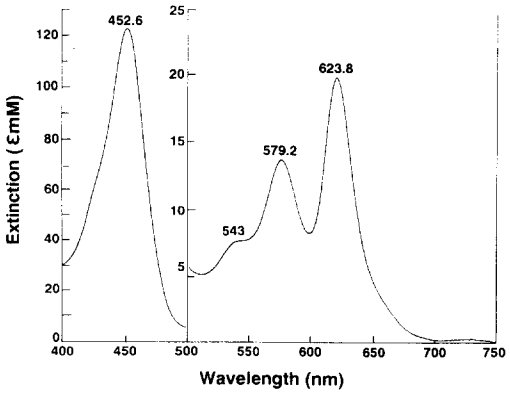

Fig. 2. Spectrum of Mg-Rchln *b*. A solution of pure Chl *b* in diethyl ether (5 ml) was evaporated to dryness under O_2-free N_2 at 60 ° C and then redissolved in 5 ml of 2% KOH in methanol in a 5 ml stoppered cuvette. This spectrum was recorded when the reaction was complete.

The presence of clear isosbestic points in both Figs. 3 and 4 indicates the occurrence of a simple reaction with no loss of reactant in side-reactions.

Knowing the extinction coefficients of Chls *a* and *b* in the original diethyl ether solution (see Experimental), the extinction coefficients of Mg-Rchlns *a* and *b* can be calculated from spectra such as those in Figs. 3 and 4 (see Table I). Both the millimolar (ϵ_{mM}) and specific (α) extinction coefficients are given at the wavelengths of the maxima of the major red peak of Mg-Rchln *a* (641.2 nm) and of Mg-Rchln *b* (623.2 nm). Using these coefficients, simultaneous equations 1 to 6 were derived to determine the concentrations of Chls *a* and *b* and total chlorophylls (Chl *a* + *b*) as their Mg-Rchln *a* and

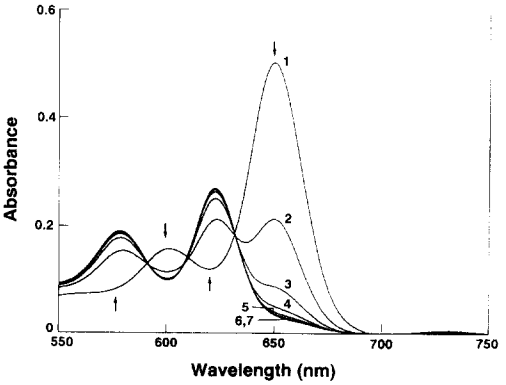

Fig. 4. Spectra of the reaction mixture during the conversion of pure Chl *b* to Mg-Rchln *b* in alkaline methanol reagent (containing 1.25 mM dithiothreitol) at room temperature. Spectrum 1 (zero time) was recorded in methanol. Spectra 2–7 were recorded at 2, 4, 7, 11, 15 and 20 min after the addition of solid dithiothreitol followed by KOH (100 mg). Spectra 6 and 7 are indistinguishable. Isosbestic points can be clearly seen.

TABLE I

Millimolar (ϵ_{mM}) and specific (α) difference coefficients for Mg-Rchlns a and b in alkaline methanol reagent

As the spectrophotometer was zeroed at 750 nm, the extinction coefficients listed below are difference coefficients as shown in column 1. Each coefficient is the mean of three separate determinations. The percentage variation about the mean (m) is presented as 100 σ/m, where σ represents the standard deviation. The percentage variation is presented (in brackets) beside the millimolar (ϵ_{mM}) coefficients but would be identical for the specific (α) coefficients which are derived from the same data.

Wavelengths appropriate to the difference coefficients (nm)	Difference extinction coefficients			
	Mg-Rchln *a*		Mg-Rchln *b*	
	(ϵ_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)	(ϵ_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)
641.2 minus 750.0	54.61 (±0.37%)	61.12	5.73 (±2.14%)	6.31
623.2 minus 750.0	14.45 (±0.70%)	16.17	19.81 (±1.03%)	21.83

b derivatives in alkaline methanol reagent. Eqns. 1 to 3 are expressed in molar terms:

$$\text{Chl } a \text{ (nmol/ml)} = 19.83\, A^{641.2} - 5.74\, A^{623.2} \quad (1)$$

$$\text{Chl } b \text{ (nmol/ml)} = 54.66\, A^{623.2} - 14.46\, A^{641.2} \quad (2)$$

$$\text{Chl } a + b \text{ (nmol/ml)} = 48.92\, A^{623.2} + 5.37\, A^{641.2} \quad (3)$$

while Eqns 4 to 6 are expressed in mass terms:

$$\text{Chl } a \text{ (}\mu\text{g/ml)} = 17.72\, A^{641.2} - 5.12\, A^{623.2} \quad (4)$$

$$\text{Chl } b \text{ (}\mu\text{g/ml)} = 49.60\, A^{623.2} - 13.12\, A^{641.2} \quad (5)$$

$$\text{Chl } a + b \text{ (}\mu\text{g/ml)} = 44.48\, A^{623.2} + 4.60\, A^{641.2} \quad (6)$$

Determination of Chls a and b converted to Mg-Rchlns a and b by extraction of biological materials with alkaline methanol reagent

In the above studies with pure Chls *a* and *b*, no allomerization was observed, even in the absence of reductant. However, allomerization occurred when discs of spinach leaves were ground in a mortar at room temperature with 2% KOH in methanol or when concentrated DMF extracts of spinach chloroplasts were added to the same alkaline reagent. Fig. 5 shows the spectral effects of allomerization on a Chl *a* and *b* mixture, namely the formation of some allomers with absorption peaks at higher wavelengths than the 640 nm peak of Mg-Rchln *a*. Spectra 1, 2 and 3 are of 40 µl of a DMF concentrate in 4 ml of methanol and alkaline methanol reagent without or with 1.25 mM dithiothreitol, respectively. Clearly, spectrum 2 is broader and flatter than spectrum 3 especially on the long-wavelength side of the peak.

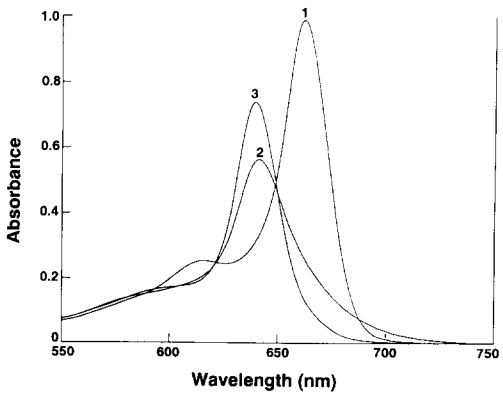

Fig. 5. Spectra of a mixture of Chls *a* and *b* in a DMF concentrate of spinach chloroplasts (40 µl) mixed with 4 ml of methanol (spectrum 1) and after conversion to Mg-Rchlns *a* and *b* in 4 ml of alkaline methanol reagent (containing 1.25 mM dithiothreitol; see spectrum 3). Spectrum 2 is of the DMF concentrate (40 µl) in alkaline methanol reagent (containing no dithiothreitol).

TABLE II

The determination of chlorophyll content and chlorophyll a/b ratios of spinach leaf discs

Spinach leaf discs (200 mm^2) were extracted with alkaline methanol reagent (see Experimental) and chlorophyll concentrations calculated using Eqns. 1–3. Discs from the same leaf were extracted with buffered 80% aqueous acetone or DMF and chlorophyll concentrations calculated as previously described [7].

Solvent system	Chlorophyll content/unit leaf area ($\mu mol/m^2$)			Chl *a*/*b* ratio (R^1) [a]
	Chl *a*	Chl *b*	Chl *a* + *b*	
Alkaline methanol reagent	232.4	71.9	304.3	3.23
Buffered 80% aqueous acetone	219.7	66.2	285.9	3.32
DMF	245.5	72.3	317.8	3.39

[a] This Chl *a*/*b* ratio (R^1), expressed in molar terms, equals $1.0156 \times r$ where r is the same ratio expressed in terms of mass.

TABLE III

Chlorophyll concentrations and chlorophyll a/b ratios in concentrated solutions of chlorophylls diluted with three solvents including alkaline methanol reagent

Concentrates of Chls *a* and *b* were prepared by extracting a spinach chloroplast pellet with DMF. An aliquot (30 μl) was diluted with 3.5 ml of alkaline methanol reagent and the chlorophyll assayed (see Experimental) using Eqns. 1–3. Similarly, 30 μl aliquots were diluted with 3.5 ml of buffered 80% aqueous acetone or DMF and the chlorophylls were determined as previously described [7].

Concentrate No.	Solvent	Chlorophyll concentrations (nmol/ml)			Chl *a/b* ratio (R^1) [a]
		Chl *a*	Chl *b*	Chl *a* + *b*	
1	Alkaline methanol reagent	12.5	3.7	16.2	3.38
1	Buffered 80% aqueous acetone	12.9	4.0	16.9	3.23
1	DMF	12.4	3.7	16.1	3.35
2	Alkaline methanol reagent	12.8	3.9	16.7	3.28
2	Buffered 80% aqueous acetone	12.3	3.9	16.2	3.15
2	DMF	12.7	3.8	16.5	3.34

[a] This Chl *a/b* ratio (R^1), expressed in molar terms, equals $1.0156 \times r$ where r is the same ratio expressed in terms of mass.

With the use of reductants to overcome the allomerization problem, it was now possible to test Eqns 1 to 6. Chlorophylls were extracted at room temperature from spinach leaf discs with alkaline methanol reagent and assayed (see Experimental), and the results (Table II) were compared with extraction and assay with buffered 80% aqueous acetone and DMF as extractants as previously described [7]. The consistency or compatability of Eqns. 1 to 6 for alkaline methanol reagent with those obtained for DMF or buffered 80% aqueous acetone [7] was tested more rigorously by assaying Chls *a* and *b* present in monomeric solution in 30 μl of two concentrates added to 3.5 ml of the three solvent systems (Table III): the results obtained with each solvent system were remarkably consistent.

It was interesting to note that when the DMF concentrate was added to alkaline methanol reagent and the reaction was performed at room temperature, isosbestic points were seen in the last 80 min of the reaction: all the Chl *b* had been converted to Mg-Rchln *b* in the prior 20 min and so the isosbestic points appeared at the wavelengths relevant to the Chl *a* reaction.

Extraction of refractory Chls a and b as Mg-Rchlns a and b from recalcitrant green algal cells of N. atomus (strain CS 183)

A suspension of growing cells of *N. atomus* was harvested by centrifugation and extracted by suspending at 60 °C for 20 min in alkaline methanol reagent as described (see Experimental). Although some chlorophyll was extracted, the pellet was still green and this colour could not be removed by repeated extraction. With a new pellet of cells, however, it was found, by replacing the alkaline methanol reagent with the aqueous alkaline methanol reagent (containing dithionite), which in addition contains 15% (v/v) H_2O, that the chlorophyll could be completely removed with a total of two or three successive extractions and the pelleted cell residue was yellow. A new set of extinction coefficients for Rchlns *a* and *b* in the 'aqueous alkaline methanol reagent' were obtained (see Table IV). Simultaneous

TABLE IV

Millimolar (ϵ_{mM}) and specific (α) difference coefficients for Mg-Rchlns a and b in aqueous alkaline methanol reagent

As the spectrophotometer was zeroed at 750 nm, the extinction coefficients listed below are difference coefficients as shown in column 1. Each coefficient is the mean of three separate determinations. The percentage variation about the mean (m) is presented as $100\ \sigma/m$, where σ represents the standard deviation. The percentage variation is presented (in brackets) beside the millimolar (ϵ_{mM}) coefficients but would be identical for the specific (α) coefficients which are derived from the same data.

Wavelengths appropriate to the difference coefficients (nm)	Difference extinction coefficients			
	Mg-Rchln *a*		Mg-Rchln *b*	
	(ϵ_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)	(ϵ_{mM}) ($l \cdot mmol^{-1} \cdot cm^{-1}$)	α ($l \cdot g^{-1} \cdot cm^{-1}$)
640.0 minus 750.0	47.93 (±0.72%)	53.61	7.18 (±1.36%)	7.92
623.0 minus 750.0	17.32 (±1.29)	19.38	17.69 (±0.84%)	19.49

TABLE V

Chlorophyll concentrations and chlorophyll a/b ratios in concentrated solutions of chlorophylls diluted with three solvents including aqueous alkaline methanol reagent

Concentrates of Chls *a* and *b* were prepared by extracting a spinach chloroplast pellet with DMF. An aliquot (80 μl) was diluted with 5.0 ml of aqueous alkaline methanol reagent and the chlorophyll assayed (see Experimental) using Eqns. 7–9. Similarly, 80 μl aliquots were diluted with 5.0 ml of buffered 80% aqueous acetone or DMF and the chlorophylls were determined as previously described [7].

Solvent	Chlorophyll concentrations (nmol/ml)			Chl *a/b* ratio (R^1) [a]
	Chl *a*	Chl *b*	Chl *a* + *b*	
Aqueous alkaline methanol reagent	7.73	2.27	10.00	3.41
Buffered 80% aqueous acetone	8.14	2.37	10.52	3.43
DMF	7.64	2.15	9.79	3.55

[a] This Chl *a/b* ratio (R^1), expressed in molar terms, equals $1.0156 \times r$ where r is the same ratio expressed in terms of mass.

equations 7 to 12 were derived from these coefficients to determine chlorophyll concentrations as their magnesium rhodochlorin derivatives in 'aqueous alkaline methanol reagent'. Eqns. 7 to 9 are expressed in molar terms:

$$\text{Chl } a \text{ (nmol/ml)} = 24.45\, A^{640.0} - 9.93\, A^{623.0} \quad (7)$$

$$\text{Chl } b \text{ (nmol/ml)} = 66.25\, A^{623.0} - 23.94\, A^{640.0} \quad (8)$$

$$\text{Chl } a + b \text{ (nmol/ml)} = 56.32\, A^{623.0} + 0.51\, A^{640.0} \quad (9)$$

Eqns. 10 to 12 are expressed in mass terms:

$$\text{Chl } a \text{ (μg/ml)} = 21.87\, A^{640.0} - 8.88\, A^{623.0} \quad (10)$$

$$\text{Chl } b \text{ (μg/ml)} = 60.14\, A^{623.0} - 21.74\, A^{640.0} \quad (11)$$

$$\text{Chl } a + b \text{ (μg/ml)} = 51.26\, A^{623.0} + 0.13\, A^{640.0} \quad (12)$$

The total chlorophyll extracted from freshly grown cells harvested from 100 ml of suspension was 261.0 nmol and the Chl *a/b* ratio was 3.08. When methanol or buffered 80% aqueous acetone were used as extractants, the total chlorophyll recovered was only about 75 and 10%, respectively. Subsequently, two further extractions, producing almost identical results, were performed on a stationary phase culture maintained for several weeks at room temperature in low light conditions. Cells from these cultures were extracted at 60 °C using aqueous alkaline methanol reagent both with and without dithionite. With reductant the total chlorophyll content per 100 ml of culture was 686.2 nmol and the Chl *a/b* ratio was 1.82, and without reductant the respective values were 709.5 nmol and 1.66. This suggests not only that the method gives reproducible results but also that when the conversion to Mg-Rchlns occurs at high temperature that oxygen concentrations in solution are reduced, thereby thwarting allomerization even in the absence of reductant. The lower Chl *a/b* ratio in the old culture must be an adaptation of the cells to a prolonged period under low light and stationary phase conditions: such cells were still recalcitrant and could not be satisfactorily extracted with either acetone or methanol.

The addition of 15% H_2O to pure methanol, without alkali, does not improve extraction of the chlorophylls, suggesting that alkali is required for the complete removal of the chlorophylls either by hydrolysing the phytyl ester bond or by cleaving the *iso*cyclic ring E or by hydrolysing components of the cell wall to render it permeable. A preliminary experiment suggested that the last explanation is correct: when *Nannochloris* cells were broken in a high-pressure cell [14] at 103.4 MPa (15000 lb/inch^2), the chlorophylls were readily removed with methanol. A hydrolysis of cell wall components would also explain the requirement for an additional 15% H_2O, since there was already sufficient water in the non-aqueous alkaline methanol reagent, presumably as a contaminant of the methanol, to convert the chlorophylls to rhodochlorins. While it is clear that breaking the cells prior to extracting with methanol is a less complicated way to remove and assay the chlorophylls of *Nannochloris* cells, many recalcitrant cells are not susceptible to mechanical breakage.

The accuracy of Eqns. 7 to 12 developed for use with aqueous alkaline methanol reagent and their consistency with the equations obtained for use with buffered 80% aqueous acetone and DMF [7] was checked by assaying the chlorophylls in 30 μl of a concentrated DMF extract of spinach leaf chloroplasts after addition to 3.5 ml of each of the three solvents: the results (Table V) again showed a considerable degree of consistency.

The addition of water to alkaline methanol reagent caused the peak maxima of Mg-Rchlns *a* and *b* to move to shorter wavelengths. At 15% H_2O the maxima for Mg-Rchlns *a* and *b* were 640.0 and 623.0 nm, respectively. Thus, the wavelength interval between the absorbance maxima of Mg-Rchlns *a* and *b*, which was shown to be 18 nm in the absence of added water (Table I), was reduced to 17 nm at water concentrations of 15% (Table V): for accurate results the peak should be accurately located and the 17 nm interval accurately

maintained when using aqueous alkaline methanol reagent. This is made considerably easier by the newest micro-computer-controlled spectrophotometers which can locate the position of a peak to the nearest 0.1 nm and then permit the operator to read absorbance at any wavelength accurate to the nearest 0.1 nm. This procedure is essential in this assay of Mg-Rchln *a* and *b* mixtures, as in the assay of Chl *a* and *b* mixtures [7], because a major problem for the accuracy of these methods is the measurement of the absorbance at the wavelength of the peak of the *b*-type derivative which always occurs on a steeply sloping segment of the spectrum of the mixture.

Discussion

The nature of allomerization reactions

While alkaline methanol converts Chls to Mg-Rchlns (see Fig. 6; Structure I), it is generally regarded as a poor solvent for the extraction and assay of chlorophylls because it is widely believed to promote allomerization leading to a complex and unassayable mixture of allomerization products. Hynninen and Assandri [3] have suggested that there are two types of allomerization reaction sequence.

One of the Hynninen and Assandri sequences is thought to involve the removal of the proton at C-13^2 by base (OH^- or OCH_3^-) to form a C-13^2 carbanion which is oxidized by molecular oxygen (air) to a carbonium ion prior to rapid reaction with hydroxyl (or methoxyl) ions to form C-13^2-hydroxy- (or methoxy-) chlorophylls [3] (see Fig. 6; Structure II). Since C-13^2-hydroxy- (or methoxy-) chlorophylls have spectra identical to that of the parent chlorophyll in the red region of the spectrum they pose no problem in the assay of chlorophylls when they are extracted and assayed as chlorophylls in the usual solvents such as buffered 80% aqueous acetone or DMF. However, this form of allomerization interferes with the assay of chlorophylls as magnesium-rhodochlorins, not only because it competes with the non-oxidative hydrolysis reactions which convert Chls *a* and *b* to Mg-Rchlns *a* and *b*, but also

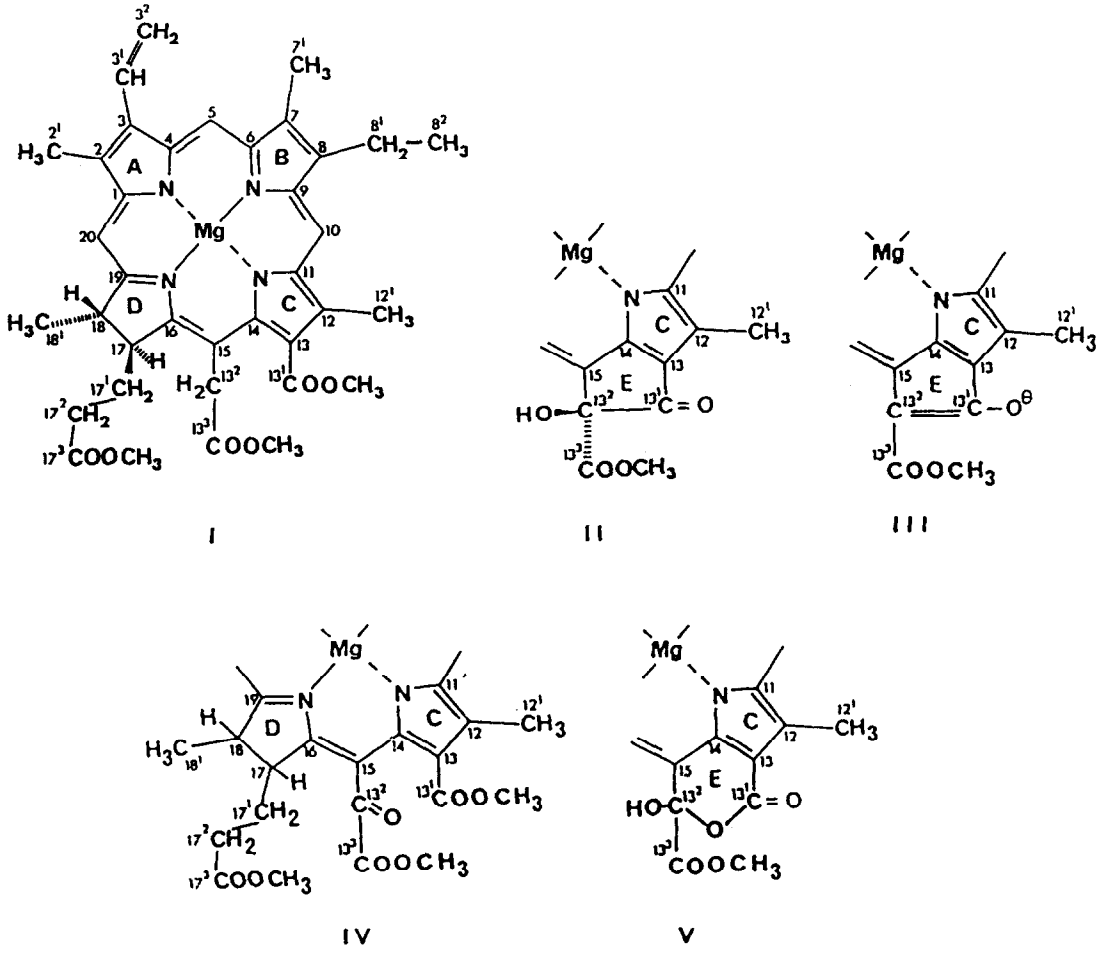

Fig. 6. The structure of magnesium rhodochlorin *a* and some intermediates and products of chlorophyll allomerization. Mg-Rchln *b* differs from Mg-Rchln *a* (I) only in its possession of a formyl group at C-7. Structures II, III and V represent the structures of C-13^2-hydroxychlorophyll, the C-13^1-enolate anion of chlorophyll and the chlorophyll cyclic hydroxylactone, respectively. Structure IV represents an intermediate in the formation of V.

because these hydrolysis products, unlike these particular allomers, have spectra different from that of the parent chlorophylls – thus the necessity to use reductants in this alkaline methanol assay to remove this oxidative side-reaction from competition.

There is a second allomerization sequence that occurs in alkaline methanol which also competes with the non-oxidative hydrolysis which produces magnesium rhodochlorins. This sequence leads to the formation of compounds possessing spectra very different to the magnesium-rhodochlorin derivatives, again making the use of reductants necessary in the alkaline methanol assay. It is proposed by Hynninen and Assandri [3] that this second type of allomerization involves the formation, in the presence of alkali of the enolate ion with an unstable double bond between C-13^1 and C-13^2 (see Fig. 6; Structure III): this double bond of the five-membered *iso*cyclic ring E is cleaved by oxygen to yield a rhodochlorin with formic and glyoxalic acid side-chains at C-13 and C-15 (see Structure IV), respectively. Subsequent ring closure to include an oxygen atom results principally in the formation of a cyclic hydroxylactone with a six-membered ring E (see Fig. 6; Structure V), but a variety of other related allomerization products are also formed [3]; the products of this second reaction sequence, like those of the first sequence, also possess hydroxy (or methoxy) groups at C-13^2.

Another free-radical chain reaction mechanism to explain the allomerization of chlorophylls has been proposed by Hynninen [15]. This mechanism involves the chlorophyll enolate anion, triplet oxygen, chlorophyll C-13^2 radicals and peroxide radicals, and a chlorophyll C-13^2 hydroperoxide. The evidence supporting this mechanism is that the formation of both C-13^2-hydroxy- or C-13^2-methoxylactone derivatives is inhibited by β-carotene, a free-radical scavenger, in the dark [15].

Selection of the appropriate reductant to inhibit allomerization during extraction of chlorophylls

Dithiothreitol and dithionite were used in many of the experiments reported in this paper and mercaptoethanol and ascorbate were used in many others not reported here. Although ascorbate also prevents allomerization and does not react with either Chl *a* and *b*, it is best avoided because it (or its oxidation product) is precipitated in methanolic solvents and adheres to the cuvette face so affecting optical properties; however, when used in extractions, the precipitate can be removed by centrifugation prior to spectrophotometry. Of the two thiol reagents, dithiothreitol was preferred to mercaptoethanol because it is more slowly oxidized under aerobic conditions and is less pungent [16,17]. However, it was found that high concentrations of dithiothreitol (12.5 mM) caused the loss of isosbestic points during the conversion at room temperature of Chl *b* to Rchln *b* presumably due to a side-reaction in which the thiol reagent reacted with the aldehyde group at C-3 forming a cyclic dithioether (mercaptal): this reaction was avoided at a low (1.25 mM) concentration when working at room temperature but not at the higher temperature of 60 °C used in the extraction procedure. Thus, it is best to avoid all thiol reductants and routinely use dithionite.

Sodium dithionite does not interfere with the conversion of Chls *a* and *b* to Mg-Rchlns *a* and *b*. Dithionite is a powerful reductant but, unlike sodium borohydride [18,19], it does not reduce the C-7 formyl group of Chl *b* or the carbonyl group found at C-13 in both Chl *a* and Chl *b*: identical spectra and identical isosbestic points were observed when pure Chls *a* and *b* were treated with aqueous alkaline methanol reagent in the absence of reductants or in the presence of dithionite. Further, the derivatives prepared in the presence or absence of dithionite behaved identically when examined by reverse-phase HPTLC on Merck RP-8 plates developed with methanol.

It is also clear from the results obtained with extractions of *Nannochloris* cells performed at 60 °C in the presence and absence of reductant (dithionite) that heating also can diminish allomerization by reducing the concentration of dissolved oxygen in the extractant; nonetheless, the use of dithionite is still recommended to prevent allomerization in manipulations occurring before heating.

Concluding remarks

Limitations on the use this assay for Chls a and b

While this assay is probably the most convenient available for Chls *a* and *b* in recalcitrant cells, it is not recommended for use with other than refractory chlorophylls because the extinction coefficients of the magnesium-rhodochlorin derivatives are approximately a half of those of the parent chlorophylls, thereby losing some sensitivity. Further, the requirement for the addition of 15% H_2O to remove refractory chlorophylls lowers the extinction coefficients approximately by yet another 5%.

In addition, this assay should not be used to assay solutions of Chls *a* and *b* that may have undergone oxidation to C-13^2 hydroxy- (or methoxy-) chlorophylls, since they will not be converted to magnesium-rhodochlorins. On the other hand, this fact may be exploited to provide the basis for a spectrophotometric estimation of any contamination by these oxidized chlorophyll derivatives.

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