Photosynthesis Research **73:** 149–156, 2002.

Minireview

The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*

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Received 4 July 2001; accepted in revised form 24 October 2001

Key words: absorption spectroscopy, accurate chlorophyll *a* and *b* determinations, algebraic correction method for Arnon's Chl determinations, chlorophyll *a/b* ratios, Daniel Arnon, LHC II, light-harvesting complex of photosytem II, G. Mackinney, magnesium atomic absorption spectroscopy, molar and specific extinction coefficients, Richard Willstätter

Abstract

Over the last half century, the most frequently used assay for chlorophylls in higher plants and green algae, the Arnon assay [Arnon DI (1949) Plant Physiol 24: 1–15], employed simultaneous equations for determining the concentrations of chlorophylls *a* and *b* in aqueous 80% acetone extracts of chlorophyllous plant and algal materials. These equations, however, were developed using extinction coefficients for chlorophylls *a* and *b* derived from early inaccurate spectrophotometric data. Thus, Arnon's equations give inaccurate chlorophyll *a* and *b* determinations and, therefore, inaccurate chlorophyll *a/b* ratios, which are always low. This paper describes how the ratios are increasingly and alarmingly low as the proportion of chlorophyll *a* increases. Accurate extinction coefficients for chlorophylls *a* and *b*, and the more reliable simultaneous equations derived from them, have been published subsequently by many research groups; these new post-Arnon equations, however, have been ignored by many researchers. This Minireview records the history of the development of accurate simultaneous equations and some difficulties and anomalies arising from the retention of Arnon's seriously flawed equations.

Abbreviations: Chl – chlorophyll; DMF – N,N′-dimethylformamide; DMSO – dimethylsulfoxide; LHC – light-harvesting complex; PS I – Photosystem I; PS II – Photosystem II

Introduction

During the last half century, plant biochemists studied the effects of different light regimes, nutrients and other growth conditions on the efficiency of various photosynthetic reactions including O_2 evolution, CO_2 fixation, or carbohydrate biosynthesis. Because of the fundamental role of chlorophylls (Chls) in photosynthesis, the rates of these reactions were often presented per unit of Chl expressed in mass or molar terms; thus, reliable assays for Chls were required. It was fortunate, therefore, that the fast and convenient simultaneous equation assays for Chls *a* and *b* of C.L. Comar and F.P. Zscheile (1942) and Daniel Arnon (1949) became available in the 1940s because, as discussed in the next section, the Chl assays of the first half of the century (see R. Willstätter and A. Stoll 1913) were slower, more difficult, and not especially accurate (cf. E. I. Rabinowitch 1945; J.H.C. Smith and A. Benitez 1955; H.H. Strain and W.A. Svec 1966).

My interest in the extraction and assay of Chls was triggered by my colleagues W.A. Thompson and P.E. Kriedemann of the CSIRO-Division of Forestry and Forestry Products, Canberra, who were studying the effects of various nutrients on the growth of Queensland Maple (*Flindersia brayleyana*); they

Figure 1. Daniel Arnon (1910–1994) at his desk in the University of California at Berkeley in 1988. His simultaneous equation assay for chlorophylls was the most frequently used after 1950. Photograph reproduced with the kind permission of Dr R. Buchanan, University of California at Berkeley.

used Chl concentrations in leaves as one indicator of plant health. They found that Chl *a* and *b* determinations in the tough leathery leaves when extracted and determined in N,N′-dimethylformamide (DMF) (Inskeep and Bloom 1985) or in methanol (Böger, 1964) differed from those obtained with aqueous 80% acetone (Arnon 1949). Further, the Chl *a/b* ratios obtained by Arnon's method were much lower than those obtained in aqueous acetone using the more accurate equations of Ziegler and Egle (1965) and H. Lichtenthaler (1987). Consequently, I decided to determine accurate extinction coefficients, both specific (α = $l \cdot g^{-1} \cdot cm^{-1}$) and millimolar ($\epsilon_{mM}$ = $l \cdot mmol^{-1} \cdot cm^{-1}$), in all three solvents to derive reliable simultaneous equations giving compatible Chl *a* and *b* concentrations and Chl *a/b* ratios (Porra et al. 1989); it should be noted here that the above definition of the specific extinction coefficient (α) used in tetrapyrrole chemistry (Smith and Benitez 1955; Porra et al. 1989) differs from that in more general use (i.e. 100 $ml \cdot g^{-1} \cdot cm^{-1}$). To achieve the required compatibility of Chl *a* and *b* concentrations and Chl *a/b* ratios, Chls *a* and *b* were freshly extracted from maize leaves and used immediately after chromatographic purification (Porra et al. 1989); some previous studies employed stored dried solid samples of Chls without further purification. The unexpected difficulty of extracting Chls from some algae, an irritating problem for some of my colleagues, was another challenge that furthered my interest in Chl extraction and assay procedures.

The derivation of simultaneous equations from molar rather than specific extinction coefficients was a rather new innovation for Chl assays at the time (Porra et al. 1989). It was inspired by the elegant work of electron crystallographers who needed to accurately determine the numbers of Chl *a* and Chl *b* molecules located on each Chl *a/b*-polypeptide of LHC II (rather than the mass of each Chl) to formulate realistic molecular models (see later section 'Molecular modeling of the major Chl *a/b* protein of LHC II').

The determination of Chls *a* and *b* and of Chl *a/b* ratios has also played an important role in investigations of how higher plants and algae adapt their photosynthetic apparatus during acclimation to new light regimes to make optimal use of ambient light intensities and qualities (see later section 'Light acclimation studies').

The derivation of the simultaneous equation method for the assay of Chls *a* and *b*

The longest and most used assay to determine the concentrations of Chls *a* and *b* in plant and algal materials was that of Arnon (1949). In this method, pigments were extracted in aqueous 80% acetone and determined in the same solvent. The concentration of each Chl was determined by measuring the extinction of the extract at the major red absorption (Q_Y) maxima of Chl *a* (∼664 nm) and *b* (∼647 nm) and inserting these values into the simultaneous equations [6] and [7] (see below). Acetone was diluted with 20% (v/v) water so that further dilution by extracted cell sap would be insignificant and thus leave the wavelength and intensity of the Q_Y maxima of Chl *a* and *b* unaffected (cf. Porra et al. 1989). Later, buffering the aqueous acetone, at pH 7.8, was introduced to minimize pheophytin formation by loss of the Mg atom in the presence of extracted metabolic acids.

Daniel Arnon (see Figure 1) used the spectrophotometric data of G. Mackinney (1941) shown in Table 1 to develop his assay. Since no other pigment extracted by these solvents, including carotenoids, in-

Table 1. Errors in the specific extinction coefficients of Mackinney (1941). The specific extinction coefficients of Chls *a* and *b* in aqueous 80% acetone obtained by Mackinney (1941) and Porra et al. (1989) are compared. The percentage errors are calculated assuming that the coefficients of Porra et al. (1989) are correct

Workers	Wavelength (nm)[a]	Chl *a*		Chl *b*	
		Spec. ext. (α)	Error	Spec. ext. (α)	Error
Porra et al. (1989)	663.6	85.95	0	10.78	0
	646.6	20.79	0	51.84	0
Mackinney (1941)	663	82.04	–4.55%	9.27	–14.01%
	645	16.75	–19.43%	45.60	–12.03%

[a]The wavelengths of the Q_Y peaks of Chls *a* and *b* are variously reported in the literature but are near 664 and 647 nm, respectively.

terfered with the red absorption of these two Chls, Arnon reasoned that the extinction (E) of these mixed Chl extracts at 663 and 645nm could be described as follows:

$$E^{663} = 82.04 \cdot [\text{Chl } a] + 9.27 \cdot [\text{Chl } b] \quad (1)$$

$$E^{645} = 45.60 \cdot [\text{Chl } b] + 16.75 \cdot [\text{Chl } a] \quad (2)$$

[Chl *a*] and [Chl *b*] represent Chls *a* and *b* concentrations expressed in $g \cdot l^{-1}$. From Equation (2),

$$[\text{Chl } a] = \frac{E^{645} - 45.60\ [\text{Chl } b]}{16.75} \quad (3)$$

By inserting Equation (3) for [Chl *a*] in Equation (1) and solving for [Chl *b*], Equation (4) is obtained:

$$[\text{Chl } b] = 0.0229 \cdot E^{645} - 0.00468 \cdot E^{663} \quad (4)$$

By inserting Equation (4) for [Chl *b*] into Equation (2) and solving for [Chl *a*], Equation (5) is obtained:

$$[\text{Chl } a] = 0.0127 \cdot E^{663} - 0.00269 \cdot E^{645} \quad (5)$$

Equations (4) and (5) are usually multiplied by 10^3 as shown in Equations (6) and (7), respectively, and the addition of Equations (6) and (7) gives Equation (8) for total Chl, designated [Chls *a* + *b*]. Equations (6), (7) and (8), which express [Chl *a*], [Chl *b*] and [Chls *a* + *b*] in $\mu g \cdot ml^{-1}$, are those published by Arnon (1949).

$$[\text{Chl } b] = 22.90 \cdot E^{645} - 4.68 \cdot E^{663} \quad (6)$$

$$[\text{Chl } a] = 12.70 \cdot E^{663} - 2.69 \cdot E^{645} \quad (7)$$

$$[\text{Chls } a + b] = 20.21 \cdot E^{645} + 8.02 \cdot E^{663} \quad (8)$$

Arnon (1949) was not the first, however, to assay Chls *a* and *b* using simultaneous equations. Previously, Comar and Zscheile (1942) assayed Chls *a* and *b* in diethylether after extraction from leaves with acetone. The Chls were displaced into diethylether by dilution with water and the ethereal phase was then washed free of acetone with more water before drying over anhydrous sodium sulphate for spectrophotometric analysis.

The simpler one-step extraction method of Arnon (1949) quickly replaced the earlier multistep technique (Comar and Zscheile 1942). Both these methods, however, rapidly supplanted the earlier and more difficult assay of Willstätter and Stoll (1913) in which Chls *a* and *b* were acidified to form phaeophytins *a* and *b*, which were treated with KOH-methanol to open the isocyclic ring and form rhodochlorins *a* and *b* (i.e. chlorin e_6 and rhodin g_7, respectively, using earlier H. Fischer nomenclature). The two rhodochlorins were transferred to diethylether, and the green rhodochlorin *a* was extracted exhaustively with 3% HCl and the red rhodochlorin *b* with 12% HCl and both were determined colorimetrically against standard solutions of known concentration. Although this assay gave low Chl *a/b* ratios (cf. Rabinowitch 1945), it produced much useful information (see section 'Light acclimation studies'). Richard Willstätter (see Figure 2) was awarded the Nobel Prize for Chemistry in 1915 for his investigations of plant pigments, especially the chlorophylls.

Later, Chls *a* and *b,* as Chls or as their pheophytins, were assayed photometrically after chromatographic separation on sucrose columns (Seybold and Egle 1938). Prior to the 1940s, Chls *a*+*b* were often assayed colorimetrically as Chls, pheophytins, or rhodochlorins against relevant standards, but with little or no allowance for differences in Chl *a/b* ratios between samples and standards. The development of simple, fast, and accurate simultaneous equation assays was, therefore, a great and much-needed advance.

Figure 2. Richard Willstätter (1872–1942), Nobel Laureate in Chemistry, 1915. In 1913, at the Kaiser Wilhelm Institut für Chemie, Berlin, Willstätter developed an assay for chlorophylls with Arthur Stoll which was in general use until 1950. © Nobel Foundation, Stockholm. Photograph reproduced with the kind permission of the Foundation.

Inaccuracy of the chlorophyll extinction coefficients used by Daniel Arnon

Over several decades, many researchers (Vernon 1960; Ziegler and Egle 1965; Delaporte and Laval-Martin 1971 a, b; Lichtenthaler 1987; Porra et al. 1989; Wellburn, 1994) discovered that Mackinney's (1941) specific extinction coefficients for Chls *a* and *b* in aqueous 80% acetone were grossly inaccurate. Mackinney's coefficients were obtained using dried solid samples of Chls *a* and *b* without further purification to remove oxidation products formed during storage. These are compared with accurate coefficients, confirmed by Mg-atomic absorption spectrometry, obtained by Porra et al. (1989) with chromatographically pure Chls (see Table 1): some of the errors are very large.

The use of alternative extractants

Alternatives to aqueous acetone for Chl extractants are DMF (N,N′-dimethylformamide), DMSO (dimethylsulfoxide) and methanol. In DMF and DMSO, as in aqueous 80% acetone, Chls *a* and *b* exhibit sharp Q_Y peaks, but DMF and DMSO are more toxic than aqueous acetone. The Q_Y absorption bands of Chls in methanol are broad and less sharp than in aqueous acetone; although an efficient extractant of Chls, methanol enhances degradation of Chls by opening the isocyclic ring, especially in alkaline conditions (Porra 1990a, 1991).

Because Chl determinations in DMF (Inskeep and Bloom 1985), methanol (Böger, 1964), and aqueous 80% acetone (Arnon 1949) were incompatible (see 'Introduction'), Porra et al. (1989) obtained accurate molar (ϵ) and specific (α) extinction coefficients for freshly prepared samples of chromatographically pure Chls *a* and *b* in these three solvents (see Table 2). The concentrations of the standard Chl *a* and *b* solutions used to determine these coefficients were verified to within an error of 1% or less, by magnesium determination using atomic absorption spectrometry (Porra et al. 1989). The simultaneous equations derived from the accurate coefficients given in Table 2 are shown in Table 3. Using the equations in Table 3, good agreement was now obtained for Chl *a* and *b* concentrations and Chl *a/b* ratios when the pigments were extracted and assayed in these three solvents (see Porra et al. 1989).

New solvents, other than aqueous acetone, were usually sought to extract Chls from difficult tissues, such as tough leathery leaves, by simple immersion for extended periods; however, finely cutting with scissors followed by grinding with extractant produced more exhaustive extraction and more satisfactory results than prolonged immersion which can cause oxidative degradation of the photolabile Chls (cf. Porra et al. 1989). Wellburn (1994) has presented accurate extinction coefficients and relevant simultaneous equations for use with various solvents including DMSO, which is sometimes used as an alternative extractant.

The following three special extractants were designed to remove Chls *a* and *b* from some green algae and marine micro-algae which were unexpectedly difficult to extract. A review of these three special extractants (Porra 1991) describes their application, the formation of derivatives and the relevant simultaneous equations for their use.

1. Aqueous 2.1 M pyridine containing 0.35 M NaOH extracts Chls *a* and *b* from regreening nitrogen-starved *Chlorella fusca* cells as their Mg-hydroxylactones formed by opening the five-membered isocyclic ring to reclose around an O atom in a six-membered ring (see Porra and Grimme 1974; Porra 1991).

Table 2. Corrected specific (α) and millimolar (ϵ_{mM}) extinction coefficients for Chls *a* and *b* in buffered aqueous 80% acetone, DMF (N,N′-dimethylformamide) and methanol. The spectrophotometer was zeroed at 750 nm so that all coefficients shown are difference coefficients between the Q_Y maximum wavelength specified and 750 nm. Each coefficient is the mean of three determinations: the standard deviations are presented in Porra et al. (1989)

Solvent	Wavelength (nm)	Difference extinction coefficients			
		Chl *a*		Chl *b*	
		Millimolar (ϵ_{mM})	Specific (α)	Millimolar (ϵ_{mM})	Specific (α)
Buffered aqueous	663.6 minus 750	76.79	85.95	9.79	10.78
80% acetone (pH 7.8)	646.6 minus 750	18.58	20.79	47.04	51.84
DMF	663.8 minus 750	79.29	88.74	12.03	13.26
	646.8 minus 750	18.62	20.84	46.49	51.23
Methanol	665.2 minus 750	71.43	79.95	20.20	22.26
	652.0 minus 750	31.65	35.42	38.55	42.48

Table 3. Simultaneous equations for the determination of Chls *a* and *b* concentrations in buffered aqueous 80% acetone, DMF and methanol using the extinction coefficients presented in Table 2

Solvent	Equations for Chl concentrations (nmol/ml)	Equations for Chl concentrations (μg/ml)
In buffered aqueous 80% acetone	$[\text{Chl } a] = 13.71\ E^{663.6} - 2.85\ E^{646.6}$ $[\text{Chl } b] = 22.39\ E^{646.6} - 5.42\ E^{663.6}$ $[\text{Chl } a + b] = 19.54\ E^{646.6} + 8.29\ E^{663.6}$	$[\text{Chl } a] = 12.25\ E^{663.6} - 2.55\ E^{646.6}$ $[\text{Chl } b] = 20.31\ E^{646.6} - 4.91\ E^{663.6}$ $[\text{Chl } a + b] = 17.76\ E^{646.6} + 7.34\ E^{663.6}$
In DMF	$[\text{Chl } a] = 13.43\ E^{663.8} - 3.47\ E^{646.8}$ $[\text{Chl } b] = 22.90\ E^{646.8} - 5.38\ E^{663.8}$ $[\text{Chl } a+b] = 19.43\ E^{646.8} + 8.05\ E^{663.8}$	$[\text{Chl } a] = 12.00E^{663.8} - 3.11E^{646.8}$ $[\text{Chl } b] = 20.78E^{646.8} - 4.88E^{663.8}$ $[\text{Chl } a+b] = 17.67E^{646.8} + 7.12E^{663.8}$
In methanol	$[\text{Chl } a] = 18.22\ E^{665.2} - 9.55\ E^{652.0}$ $[\text{Chl } b] = 33.78\ E^{652.0} - 14.96\ E^{665.2}$ $[\text{Chl } a + b] = 24.23\ E^{652.0} + 3.26\ E^{665.2}$	$[\text{Chl } a] = 16.29\ E^{665.2} - 8.54\ E^{652.0}$ $[\text{Chl } b] = 30.66\ E^{652.0} - 13.58\ E^{665.2}$ $[\text{Chl } a + b] = 22.12\ E^{652.0} + 2.71\ E^{665.2}$

2. Aqueous 85% methanol containing 2% KOH and 1.5 mM sodium dithionite extracts, Chls *a* and *b* from *Nannochloris atomus* cells as Mg-rhodochlorins *a* and *b* (i.e., Mg-chlorin e_6 and Mg-rhodin g_7) formed by opening the isocyclic ring (Porra 1990a). Dithionite prevents Mg-hydroxylactone formation.

3. Aqueous 85% methanol containing 1.5 mM sodium dithionite extracts Chls unchanged from *Nannochloris atomus* cells (Porra 1990b); presumably, the reductant also cleaves disulfide bridges to relax cell-wall proteins (cf. Thompson and Preston 1967, 1968) and render the cells permeable to methanol.

Correction of data obtained by use of the Arnon simultaneous equations

Despite the frequent publication over many decades of more accurate extinction coefficients and simultaneous equations for Chls *a* and *b* (Vernon 1960; Ziegler and Egle 1965; Delaporte and Laval-Martin 1971 a, b; Lichtenthaler 1987; Porra et al. 1989; Wellburn 1994), these more reliable post-Arnon equations were largely ignored and the Arnon equations retained. Perhaps the magnitude of the errors involved in the Arnon method was not fully appreciated. Perhaps some researchers were more interested in trends than in absolute values for either Chl concentrations or Chl *a/b* ratios

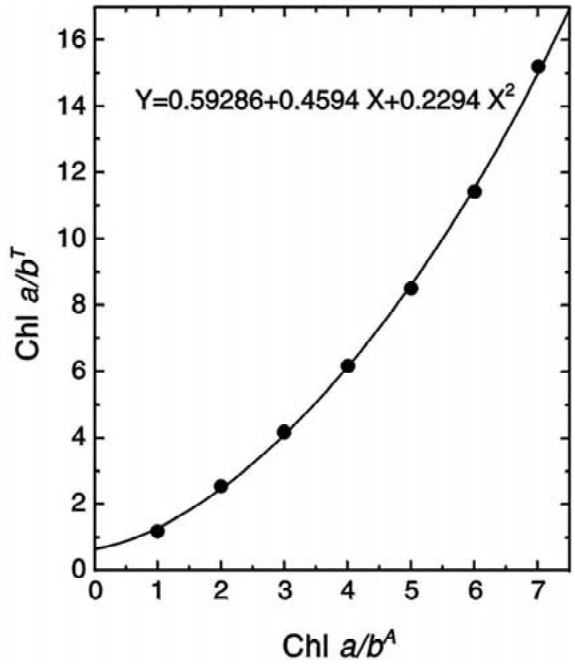

Figure 3. Using the Mackinney's extinction coefficients (see Table 1), the extinction values at the red Q_Y absorption peaks of Chls *a* and *b* were calculated for hypothetical solutions of Chl *a* and *b* in buffered aqueous 80% acetone with Arnon ratios, Chl a/b^A, from 1.0 to 7.0. These values were inserted into the appropriate equations of Table 3 to obtain true Chl *a* and *b* concentrations and hence true ratios, Chl a/b^T. The quadratic equation which best fits the curve is shown: it was determined using the Microcal Origin Program (Version 4.0).

and, therefore, retained the Arnon method to permit comparison between current and previous results. To remove this relativity obstacle, a quick and precise algebraic method was developed to correct Chl *a* and Chl *b* determinations obtained by Arnon's equations without reference to the original spectrophotometric data (Porra et al. 1989; Porra 1991).

The correction method was developed by plotting Arnon Chl *a/b* ratios, Chl a/b^A, against true ratios, Chl a/b^T (see Figure 3). The quadratic equation which best fits the curve is shown in Figure 3 and again as Equation (9):

$$\text{Chl}a/b^T = 0.593 + 0.459\cdot(\text{Chl}a/b^A) + 0.229\cdot(\text{Chl}a/b^A)^2 \quad (9)$$

Calculations for conversion of Chl a/b^A to Chl a/b^T (see legend to Figure 3) showed that the true total Chl, designated $[\text{Chl } a + b]^T$, was always 89.5% of the total Chl, $\text{Chl}[a + b]^A$, calculated by Arnon's method [see Equation (10)]:

$$[\text{Chls } a + b]^T = 0.895[\text{Chls } a + b]^A \quad (10)$$

Thus, having obtained Chl a/b^T from Figure 3 or Equation (9) and by calculating $[\text{Chl } a + b]^T$ from Equation (10), the true Chl *a* and *b* concentrations, designated $[\text{Chl } a]^T$ and $[\text{Chl } b]^T$, can be calculated using Equations (11) and (12):

$$[\text{Chl } a]^T = \frac{[\text{Chl } a + b]^T \cdot \text{Chl } a/b^T}{(\text{Chl } a/b + 1)} \quad (11)$$

$$[\text{Chl } b]^T = [\text{Chls } a + b]^T/(\text{Chl } a/b^T + 1) \quad (12)$$

Figure 3 shows that the quotient of Chl $a/b^T \div$ Chl a/b^A increases from 1.17 to 1.52 to 2.17 for Chl a/b^A values of 1.0, 4.0 and 7.0, respectively; thus, the higher the Arnon ratio the greater the error. This has important consequences (see later section on 'Light acclimation studies').

Some consequences of the continued use of the Arnon equations

Molecular modeling of the major Chl a/b protein of LHC II

Accurate Chl *a* and *b* determinations were required for the electron crystallography studies of light-harvesting complex (LHC) II by Werner Kühlbrandt and his group. Their goal to build a realistic molecular model of this Chl *a/b*-protein complex required that they know the precise number of Chl *a* and *b* molecules present in each pigment–protein molecule. Using Arnon's assay, Butler and Kühlbrandt (1988) and Kühlbrandt and Wang (1991) found 15 Chl molecules per LHC II protein molecule with a Chl a/b^A ratio of 1.15, which suggested 8 Chl *a* and 7 Chl *b* molecules per pigment protein. The atomic model derived from their electron-crystallography results (Kühlbrandt and Wang 1991) indicated only 12 Chl molecules present and, with a Chl a/b^A ratio of 1.15, they deemed them to be 7 Chl *a* and 5 Chl *b*.

Later, using more accurate equations (Porra et al. 1989), Kühlbrandt et al. (1994) found 14 Chls per pigment–protein molecule and a Chl a/b^T of about 1.3, suggesting 8 Chl *a* and 6 Chl *b* per protein. Disappointingly, the more accurate equations of Porra et al. (1989) only reduced the Chl to protein ratio from 15 to 14. Considering the enormity of the errors in Mackinney's coefficients (see Table 1), it is unlikely that sufficient error remains in those of Porra et al. (1989) to permit elimination of a further 2 Chl molecules to lower the Chl/protein ratio to 12. As suggested by

Kühlbrandt et al. (1994), the discrepancy between the experimentally determined Chl content and the number of Chl molecules observed in the atomic model is probably caused by 2 Chl molecules remaining non-specifically bound to the LHC II protein throughout purification and crystallization: this discrepancy of 2 Chl molecules still remains in their more recent work (Rogl and Kühlbrandt 1999).

Light acclimation studies

A more dramatic anomaly arising from retention of Arnon's equations was encountered in investigations of chloroplast acclimation to ambient light intensities. Low-light (shade) plants contain more chlorophyll per chloroplast and have lower Chl *a/b* ratios than high-light (sun) plants (Anderson 1986); incidentally, this had also been established in 1913 by Willstätter and Stoll (cf. Rabinowitch 1945). In both shade and sun plants, Arnon's Chl a/b^{A} ranges and those of Willstätter and Stoll (1913) were low. The typical Chl a/b^{A} range for shade plants is about 1.6–2.2 which, using Figure 3, becomes a true Chl a/b^{T} range of 2.0–2.8: for sun plants the typical Chl a/b^{A} range is approximately 2.6–3.4 which becomes a Chl a/b^{T} range of 3.5–4.9 using Figure 3.

These changes in Chl *a/b* ratios in shade and sun plants reflect the adaptation of the chloroplast to prevailing light intensity through regulation of the amount of Photosystem I (PS I) relative to Photosystem II (PS II) and the size and composition of the light-harvesting complexes (LHCs) of each photosystem. Shade plants, relative to sun plants, contain more Chl in larger grana (Anderson 1986). Relative to PS I, shade plants have fewer PS II but with very large light-harvesting antennae compared to those of sun plants. The most abundant Chl *a/b* protein, LHC II, in shade-plant chloroplasts has a very low Chl a/b^{A} of 1.15 (i.e. Chl a/b^{T} of 1.3); thus, shade plants have lower Chl *a/b* ratios than sun plants which contain less Chl and possess only small antennae located in more numerous but smaller grana (Anderson 1986).

Sane et al. (1970) and Andersson et al. (1976) separated PS I- and PS II-enriched fractions from fragmented chloroplasts. With Arnon's method, the Chl a/b^{A} range for the PS I-enriched fraction was approximately 6.0–6.5 which, using Figure 3, became a true Chl a/b^{T} range of 11.4–13.4. The PS II-enriched fractions have a Chl $a/b^{A} < 3.0$ (Andersson et al. 1976) which, using Figure 3, becomes Chl $a/b^{T} < 4.2$. Interestingly, the much less abundant light-harvesting Chl *a/b* protein of PS I has a Chl a/b^{A} of 3.6 (Thornber 1986). which corresponds to a Chl a/b^{T} of 5.25.

All these corrected Chl *a/b* values, especially for the PS I-enriched fraction, clearly indicate the need to specify whether assays were performed by the Arnon method or a more accurate post-Arnon assay (cf. Vernon 1960; Ziegler and Egle 1965; Delaporte and Laval-Martin 1971a, b; Lichtenthaler 1987; Porra et al. 1989; Wellburn 1994).

Defining the limitations of the simultaneous equation assay for Chls a *and* b

The contribution of Chl *b* at 647 nm is difficult to measure on a steeply sloping Chl *a* spectrum when the Chl *a/b* ratio is high; thus the Chl content of PS I is probably better assayed using the Chl *b*-oxime method of Ogawa and Shibata (1965). In this assay, the extinction at 666 nm is read before and after the addition of 40% aqueous hydroxylamine (pH 4.0) to the Chls extracted in methanol; this converts the 7-formyl group of Chl *b* to form Chl *b*-oxime which, like Chl *a*, has a Q_Y peak at 666 nm. The derivation of the relevant simultaneous equations to determine both Chl *a* and *b* are described by Ogawa and Shibata (1965). When the Chl a/b^{A} ratio exceeds 7.0, which is equivalent to a Chl a/b^{T} value of about 15 (see Figure 3), the Chl *b*-oxime assay or, alternatively, a spectrofluorimetric method (see Talbot and Sauer 1997) should be used.

Concluding remarks

It appears contrary to reason that so many researchers continue to use Arnon's equations (1949) after more accurate post-Arnon equations have become available; continuing with Arnon's assay to retain relativity between their earlier and current results was no longer sufficient reason after the simple algebraic method to correct the earlier results of Arnon's assays became available (Porra et al. 1989).

As noted above, the error in Arnon's ratios (Chl a/b^{A}) increases as the ratios become higher. The true Chl *a/b* ratio range of 11.4–13.4 for PS I-enriched fractions is about double the 6.0–6.5 range calculated by the Arnon method (see earlier section on 'Light acclimation studies'). This starkly emphasizes the absurdity of retaining an old inaccurate assay and clearly indicates the potential for even more confusion that its further retention could generate.

Acknowledgments

I thank Govindjee for the invitation to write this minireview, and I am most grateful to Dr W.S. Chow for calculating the quadratic equation best describing the relationship between Chl a/b^{T} and Chl a/b^{A} shown in Figure 3 and Equation (9).

I thank the CSIRO-Division of Plant Industry, Canberra, for an Honorary Fellowship and the Sonderforschungsbereich (Projekt 533) for financial assistance during my visit to the Botanisches Institut, Ludwig-Maximilians Universität, München. I thank the Division and Institut for use of their communications, computer and library facilities during the preparation of this article. I also thank Prof. Hugo Scheer for helpful discussions.

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